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Use of the Comet Assay to study the role of MGMT, MMR and p53 in the repair of alkylating DNA damage

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Professora Doutora Cristina Pereira-Wilson

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DECLARAÇÃO

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ABSTRACT

Alkylating agents are one a major class of mutagens contributing to DNA damages and carcinogenesis. However, as alkylating agents are powerful inducers of DNA damage related cell death, and are commonly used in the chemotherapy. O^6-methylguanine (O^6meG) and O^6-chloroethylguanine (O^6ClethG) are the most cytotoxic lesions caused by Temozolomide (TMZ) and 1,3-bis-(2-chloroethyl)-1-nitrosourea (BCNU), respectively. MGMT (O^6-methylguanine-DNA methyltransferase) repair protein directly reverses O^6alkylG lesions and, consequently, it is considered as a prognostic marker of resistance in cancer cells exposed to alkylating agents.

Therefore, expression levels of this protein predict cancer cell susceptibility and the success of therapy. However, it has been observed that not all tumors with low MGMT activity respond to alkylating agents with increased cell death. Mismatch repair (MMR) system and functional status of p53 are also two most relevant factors in this response.

The aims of this project were to study the genotoxic effects of alkylating drugs as detected by the CoMeth assay, and to determine the dependence of a functional MMR system and p53 in the sensitivity of cells to treatment. Thus, using a proliferating MMR-proficient cancer cell line Caco-2, we report that CoMeth assay allows the qualitative evaluation of O^6meG lesions as well as O^6ClethG, after their conversion to strand breaks. To determine whether the DNA damages (comet assay) and cell death (nuclear condensation assay) induced by (TMZ) and (BCNU) agents are mediated by MMR we used a MMR-deficient colon HCT116 cell line. We observed that even though MMR status seemed to be determinant for cells' sensitivity to TMZ, this was not applied for the case of BCNU. Resistance to the O^6-chloroethylating agent involves other mechanisms that are independent of functional MMR system. We found that induction of DNA damages by BCNU was independent of p53 status, however p53-wt cells showed more pronounced apoptosis compared with p53-null cells.

Altogether, our data show that functional MMR system is required for genotoxicity (apoptosis) induced by TMZ. For BCNU, we show that the efficiency on cancer cells is not dependent on the MMR status but only the activity of MGMT. In p53-wt cells, cell death by BCNU was more pronounced. This project also demonstrated the value of the CoMeth assay as a tool for the study of anticancer chemotherapeutic drugs.

RESUMO

Agentes alquilantes são uma das maiores classes de mutagénicos contribuindo para os danos no DNA e para a carcinogénese. Contudo, como os agentes alquilantes são indutores potentes de danos no DNA relacionados com a morte celular eles são frequentemente usados na quimioterapia. O^6-metilguanina (O^6meG) e O^6-cloroetilguanina (O^6-cletG) são as lesões mais citotóxicas causadas pela Temozolomida (TMZ) e 1,3-bis-(2-cloroetil)-1-nitrosureia (BCNU), respetivamente. MGMT (O^6-metilguanina-DNA metiltransferase) repara a proteína e reverte diretamente as lesões O^6-alquilG e, consequentemente, é considerado um marcador de prognóstico de resistência em células cancerígenas expostas a agentes alquilantes.

Desta forma, os níveis de expressão desta proteína preveem a suscetibilidade das células cancerígenas e o sucesso da terapia baseada em agentes alquilantes. Contudo, tem sido observado que nem todos os tumores com baixa actividade da MGMT respondem a agentes alquilantes com morte celular aumentada. O sistema de reparação Mismatch repair (MMR – do inglês mismatch repair) e o estado funcional do p53 são também dois dos fatores mais relevantes nesta resposta.

Os objetivos deste projeto foram estudar os efeitos genotóxicos de drogas alquilantes, sendo detetadas pelo ensaio do Cometa e determinar a dependência do estado funcional do sistema Mismatch repair e do p53 na sensibilidade às drogas alquilantes. Desta forma, usando uma linha celular cancerígena proliferativa Caco-2, com um sistema MMR eficiente, demonstramos que o ensaio do Cometa permite uma avaliação qualitativa das lesões O^6meG bem como as O^6-cletG, depois da sua conversão em quebras na cadeia de DNA. Para determinar se os danos no DNA (ensaio do cometa) e morte celular (ensaio de condensação nuclear) induzidos pelos agentes metilantes (TMZ) e cloroetilantes (BCNU) são mediados por reparação MMR usamos a linha celular HCT116 deficiente no sistema MMR. Observamos que apesar do estado MMR parecer ser determinante para a sensibilidade das células à TMZ, este facto não foi observado para o caso do BCNU. A resistência ao agente O^6-cloroetilante envolve outros mecanismos que são independentes do sistema funcional MMR. Verificamos que a indução dos danos no DNA pelo BCNU foram independentes do estado do p53, contudo as células com p53 ativo apresentaram uma apoptose mais pronunciada comparado com as células com p53 inativo. De um modo geral os nossos resultados mostram que o sistema funcional MMR é necessário para a genotoxicidade (apoptose) induzidos pela TMZ. Para o BCNU, mostramos que a eficiência nas células cancerígenas não é dependente do estado do MMR mas apenas da actividade da MGMT. Nas células com p53 ativo a morte celular induzida pelo BCNU foi mais pronunciada.

Este estudo também demonstrou o papel do ensaio do cometa como ferramenta no estudo de drogas anticancerígenas.

CONTENTS

ABBREVIATIONS LIST

5-FU	5-Fluorouracil
8-oxoG	8-Oxo-7,8-dihydroguanine
ACNU	Nimustine
ALKBH	AlkB homologue
ALS	Alkali-labile sites
AP	Abasic sites
APC	Adenomatous polyposis coli
APE1	Apurinic/apyrimidinic endonuclease
ASR	World age-standardized incidence rate
ATM	Ataxia telangiectasia mutated
ATR	Ataxia telangiectasia-rad3-related kinase
ATRIP	ATR-interacting protein
BCNU	1,3-bis(2-chloroethyl)-1-nitrosourea
BER	Base excision repair
BSA	Bovine serum albumin
CHK1	Checkpoint kinase 1
CHK2	Checkpoint kinase 2
CIN	Chromossomal instability
CRC	Colorectal cancer
Curc	Curcumin
DDR	DNA damage response
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DSB	Double strand break
EGCG	(-)-epigallocatechin-3-gallate
EndoIII	Endonuclease III
FA	Fanconi anemia
FAP	Familial adenomatous polyposis coli
FBS	Fetal bovine serum
FPG	Formamidopyrimidine DNA glycosylase
GGR	Global genomic repair
hMLH1	human MutL homolog 1
hMSH2	human MutS homolog 2
HNPCC	Hereditary non-polyposis colorectal cancer
HR	Homologous recombination
ICL	Interstrand crosslink
IDL	Insertion-deletion loop
KRAS	v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog

L	Luteolin
LMP	Low melting point agarose
LP-BER	Long-patch BER
MGMT	O^6-methylguanine-methyltransferase
MMR	Mismatch repair
MMS	Methyl methanesulfonate
MNNG	*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine
MNU	*N*-methyl-*N*-nitrosourea
MPG	N-methylpurine-DNA glycosylase
MSI	Microsatellite instability
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
N1-O^6-ethanoG	N1-O^6-ethanoguanine
NER	Nucleotide excision repair
NHEJ	Non-homologous end joining
NMP	Normal melting point agarose
O^4-meT	O^4-methylthymine
O^6-BG	O^6-benzylguanine
O^6-ClethG	O^6-chloroethylguanine
O^6meG	O^6-methylguanine
OA	Oleanolic acid
OGG1	8-Oxoguanine glycosylase
PBS	Phosphate buffered saline
PI3K	Phosphatidylinositol 3-kinase
PMSF	Phenylmethylsulfonyl fluoride
PTEN	Phosphatase and tensin homolog deleted on chromosome 10
PVDF	Hybond-P polyvinylidene difluoride membranes
Q	Quercetin
R	Rutin
RA	Rosmarinic acid
Resv	Resveratrol
ROS	Reactive oxygen species
RPA	Replication protein A
SB	Strand break
Sil	Silibinin
SP-BER	Short-patch BER
SSB	Single strand break
TCR	Transcription coupled repair
TGF β	Tumor Growth Factor b
TMZ	Temozolomide
TP53	Tumor protein p53
UA	Ursolic acid

1. INTRODUCTION

Cancer are the most health problem and are the leading cause of death in economically developed countries. This problem arises as a consequence of population aging and adoption of cancer-associated behaviors including smoking, alcohol and diets (Jemal et al., 2011). Even though the advance, over the years, in treatment strategies, such as surgery, radiotherapy and chemotherapy, it is estimated that, in 2008, 12.7 million new cancer cases and 7.6 million cancer related deaths occurred (Ferlay et al., 2010).

The process of carcinogenesis is highly complex and comprise a set of interactions between molecular (genetic and epigenetic) and environmental factors. Tumorigenesis is originated through one or more mutations that occur in a cell, giving them a selective advantage by enabling it to grow under conditions of limitation of nutrients, oxygen and/or growth factors. These cells, through a selection process, are capable of multiplying and invading large regions of the organism. These abnormal cellular features acquired during tumor development are known as the hallmarks of cancer (Hanahan and Weinberg, 2011). This ten proposed hallmarks including sustained proliferative signaling, resistance to cell death, replicative immortality, stimulation of angiogenesis, evasion from immune destruction, genome instability and mutation, deregulation of cellular energetics, promotion of tumor inflammation and evasion from growth suppressors (Figure 1.1).

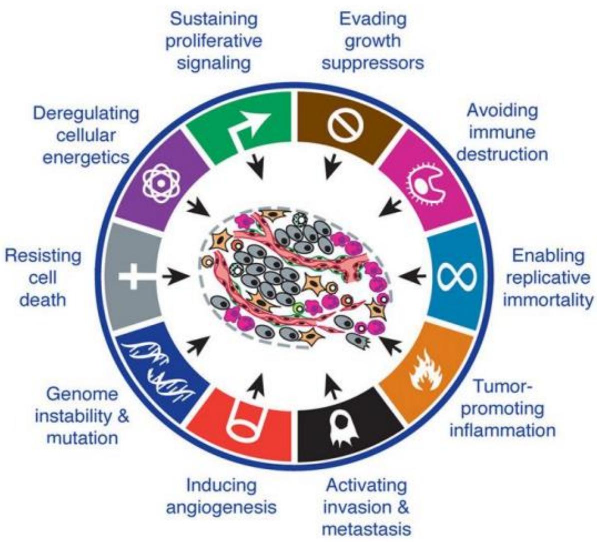


Figure 1.1 – The tem hallmarks of cancer proposed in 2011 by Hanahan and Weinberg (Adapted from (Hanahan and Weinberg, 2011).

Through to genetics analysis of tumors it was possible identify two types of genes: the oncogenes, which are the capacity to potentiate the tumorigenesis, and tumor suppressor genes, that are responsible for protecting the cells against any event that promote the transformation of a normal cell into a neoplastic cell.

Studies in several cancers revealed that abnormalities in twelve pathways or regulatory processes can lead to cancer progression such as: DNA damage control, apoptosis, regulation of G1/S phase progression, cell adhesion, c-Jun N-terminal kinase signaling, integrin signaling, KRAS signaling, small GTPase-dependent signaling, regulation of invasion, Wnt/Notch signaling, TGF-β signaling and hedgehog signaling (Gerstung et al., 2011; Jones et al., 2008).

Nonetheless, only the broad knowledge of pathways which has a role in tumorigenesis, will allow the design of new therapies that can offer a higher survival for patients.

1.1. Colorectal carcinoma (CRC)

Due to the high regeneration ability of the intestinal epithelium, processes such as proliferation and apoptosis are very important to maintain homeostasis, and consequently deregulation of these mechanisms in addition of other mutations can lead to colorectal cancer (CRC) development (Araújo et al., 2011).

Worldwide, colorectal cancer (CRC) is the third most common cancer in men and the second in women. According to GLOBOCAN 2008, the world age-standardized incidence rate (ASR) of this cancer is substantially higher in men than in women (20.3 vs. 14.6 per 100,000 people, respectively).This tendency is also verified in Europe (37.4 in men vs. 23.9 in women) and in Portugal (40.6 in men vs. 24.1 in women). These tumors are more incident in developed countries than in developing countries. This cancer is also characterized by high mortality rates (Globocan, 2008).

The CRC tumors are divided in two subclasses: hereditary or sporadic. The most common type of CRC is the sporadic cancer and, in this case, risk factors include lifestyle (e.g. physical inactivity and obesity), environmental factors (such as excessive alcohol consumption and smoking, high consumption of red meat) and age and most patients being over 50 years old (Berlau et al., 2004; Jemal et al., 2011; Souglakos, 2007). Only around 20% of the total cancers are hereditary, and the most common are the Lynch syndrome (also called as hereditary non-polyposis colorectal cancer (HNPCC)) that is caused by germiline mutations in mismatch repair (MMR) system. The loss of functional MMR system in Lynch syndrome patients occurs due to the epigenetic silencing of genes involved in this pathway (by DNA methylation) as well as by mutations in MMR genes (Markowitz and Bertagnolli, 2009).

The syndrome familial adenomatous polyposis (FAP) is the other example of hereditary colorectal cancer and results from germiline mutations in *APC* (adenomatous polyposis coli) gene (Souglakos, 2007).

The accumulation of mutations in several genes such as tumor suppressor genes, and oncogenes occurs often in CRC but also in other tumor types (Futreal et al., 2004). In CRC, a sequence of events has been described in several genes that promote the development of CRC. The mutation in the tumor suppressor gene *APC*, which is not only responsible for hereditary cancer but also plays a role in sporadic tumors, represents the most common mutation in the early phase of CRC development. This gene is responsible for controlling the WNT signaling pathway and controls specific proteins involved in this signaling such as β-catenin. Loss of function mutations in the *APC* result in the nuclear translocation and accumulation of β-catenin where it will be promote the activation of several transcription factors involved in proliferation and differentiation processes in the intestinal crypt epithelial cells. Other frequent mutation occur in the *KRAS* gene and happens during later phases of tumor progression (Vogelstein and Kinzler, 2004). Mutations that affect *KRAS* lead to unregulated cell proliferation and, consequently, the malignant transformation since leads to the constitutive activation of this pathway. The mutation in *TP53* gene is another preponderant alteration found in CRC and occurs during the late phase of CRC development. This mutation occurs in approximately 50% of CRC. The p53 transcription factor is a transcriptional regulator of genes that are involved in cell cycle, regulating apoptosis and DNA repair. As a consequence, p53 mutations facilitate ilimited growth and apoptosis evasion (Fearon, 2011).

As referred above, genetic alterations in genes that encode enzymes belonging to MMR system also have a crucial impact in CRC. Nearly 10-15% of sporadic cancers exhibit mutations in MMR genes. Malignant transformation is facilitated by the presence of these mutations since this allows the accumulation of DNA damages. The genetic instability detected in CRC may arise by from two pathways: chromosomal instability (CIN) and microsatellite instability (MSI). The CIN pathway causes allelic losses, chromosomal translocations and amplifications (Redston, 2001; Söreide et al., 2006). Microsatellite instability pathway, MSI, occurs in approximately 15% of CRC. Microsatellites are frequently found in whole DNA sequence and are constituted by repeating sequences of nucleotides. Therefore, MSI is a condition in which a microsatellite allele loss or gains repeated sequences in chromosomal regions. MSI is due to mutational or epigenetic silencing of MMR genes (Fearon, 2011). This way, the detection of defects in MMR system is one of the most promising biomarkers in CRC. MSI has been found in several cases of sporadic cancer where it is mainly due to epigenetic silencing caused by methylation of CpG islands presents in *MLH1* promoter gene (approximadetly 95% of the cases) (Markowitz and Bertagnolli, 2009). In turn, in hereditary colorectal cancer the instability in microsatellite sequences is caused, frequently, by mutations in some mismatch repair genes such as, *hMSH2*, *hMLH1* and *hMSH6* (Söreide et al., 2006) (Figure 1.2). With regard to MSI CRC may be subdivided in three MSI phenotypes: microsatellite stable (MSS, none sequences affected), MSI high (MSI-H, at least 40% of loci are affected) and MSI low (MSI-L, a single locus is affected) (Fearon, 2011).

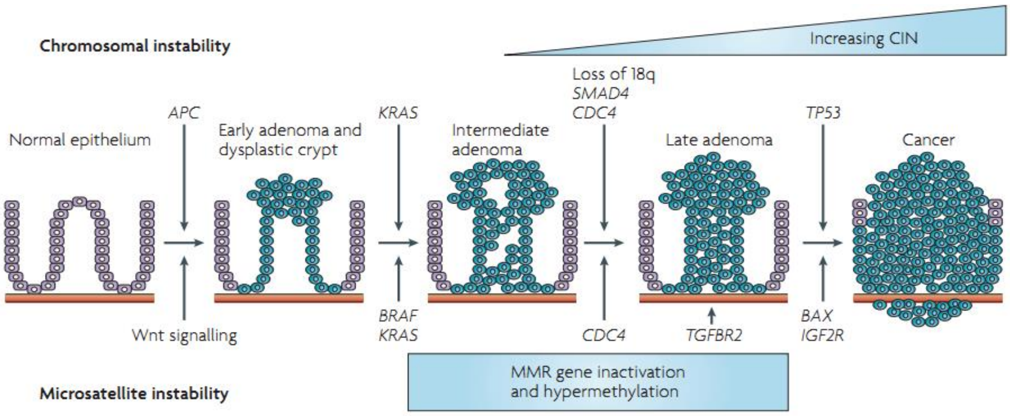


Figure 1.2 - Schematic representation of some genetic alterations during the evolution of colorectal cancer (Adapted from (Walther et al., 2009).

In CRC, the survival rates have significantly improved since there has been an increase in the knowledge about the prevention/ risk factors, early detection and therapy.

It has been estimated that it is necessary a long period of time (approximately 10-17 years) for colon cancer development because requires several mutations, as referred above. This lag time provides an opportunity to early detection and for development of prevention strategies (Chen and Huang, 2009).

In terms of treatment of CRC, great advances have been made that allow increasing the survival from 6 months to 2 years. After surgical removal of the tumor, the treatment is mainly based on chemotherapy with 5-Fluorouracil (5-FU), however some resistance to this drug have been reported in several cases. Therefore, to overcome this problem other drugs mainly irinotecan and oxaliplatin are used in combination with 5-FU. More recently, monoclonal antibodies (such as, cetuximab and panitumumab) have been developed with the aim to inhibit specific targets involved in tumoral proliferation (for instance, epithelial growth factor receptor - EGFR) (Bhushan et al., 2009; Davies and Goldberg, 2008).

1.2. DNA damages and genomic stability

The integrity and stability of the DNA double helix is essential for the normal function of organisms. However, DNA is constantly exposed to modifications from a variety of damaging agents, of both endogenous and exogenous sources. The majority of endogenous damages is caused by cellular metabolites (such as reactive oxygen species – ROS and replication errors), while exogenous sources of DNA damaging agents comprise ionizing radiation, toxins, ultraviolet light and pollutants or, in cancer patients, chemotherapeutic drugs.

The major type of alterations in DNA induced by alkylating agents may cause single- or double-strands DNA breaks (SSB and DSB, respectively), crosslinks (intrastrands or interstrands - ICls) formation of apurinic/apyrimidinic (AP) lesions (also known as abasic sites), base modifications (such as alkylated bases), and DNA-protein crosslinks (Figure 1.3) (Maynard et al., 2009; Spry et al., 2007). Cell death and aging are examples of some events that result from these alterations in the genome.

In mammalian cells, there are some cellular responses that may be activated by DNA damages, referred above, in order to remove the toxic effects of the anticancer agents. These responses include: 1) activation of DNA checkpoints for modulation of cell cycle progression allowing the repair of the damages and prevents the transmission of the lesion; 2) induction of cell death allowing the elimination of damage or deregulated cells; 3) transcriptional response causing modifications in transcription levels of certain genes (verifying the up- or downregulation of genes) and 4) induction of DNA repair pathways for complete removal of DNA damages. Collectively, these cellular responses determine whether cell survive or goes into cell death by apoptosis (Madhusudan and Middleton, 2005).

Therefore, repair and protection of DNA are two key mechanisms very important in the prevention of several pathologies including cancer (Sancar et al., 2004; Spry et al., 2007).

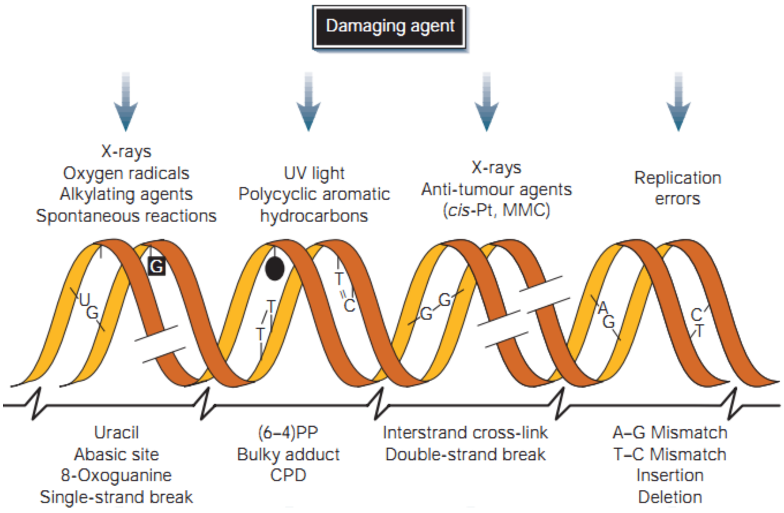


Figure 1.3 – Representation of common DNA damaging agents and respective DNA lesions induced by these agents (Adapted from (Hoeijmakers, 2001).

1.3. Alkylating agents

Even though oxidative damages are the most studied, DNA alkylation damages have also a critical role in cancer development and treatment. Alkylating agents represent a ubiquitous family of chemicals that react with several biomolecules by transfer of alkyl groups causing alteration in the structure and the function of these molecules.

Alkylating agents may be present either in the environment or inside cells. Potential exogenous sources comprise contaminants (such fuel combustion, tobacco smoke) and the heterocyclic amines existent in food, while endogenous sources derived from such as subproducts of oxidative stress (Drabløs et al., 2004; Fu et al., 2012; Wirtz et al., 2010).

Alkylating agents are able to react with oxygen (O) and ring nitrogen (N) present in DNA nitrogenous bases. DNA lesions created by alkylating agents are determined by their: chemical reactivity (S_N1-type or S_N2-type nucleophilic substitution), amount of reactive sites (designated as monofunctional or bifunctional), alkyl group added (methyl, chloroethyl, etc) and the DNA substrate (single or double stranded) (Fu et al., 2012).

The range of DNA lesions that can be created by the action of alkylating agents include, *O*-alkylated lesions (e.g. O^6-methylguanine (O^6meG) and O^4-methylthymine (O^4meT)) and *N*-alkylated lesions (e.g. N^7-methylguanine (N^7meG) and N^3-methylguanine (N^3meG)).Even though alkylation in the atoms of oxygen are produced in much smaller amounts than *N*-alkyl lesions, *O*-alkyl lesions have a great biological significance since theses lesions, during DNA replication, can cause highly genotoxic and mutagenic effects. Generally, *N*-alkylations are less mutagenic since are rapidly removed by base excision repair (BER) or the AlkB homologue (ALKBH) (Boysen et al., 2009; Kondo et al., 2010b). The elevated reactivity of the N-atoms leads to that this alkylation represents over 80% of alkylated bases. For instance, N^7meG is a very common methylation comprising 60-80% of the total alkylation lesions in molecule of DNA. N^3meA and N^3meC comprise 10-20% of the total methyl damages. Of total DNA adducts, O^6-methylguanine is present only in 8% (after methylnitrosourea exposure, MNU) and in 0.3% (after methyl methanesulfonate exposure, MMS). O^4meT is less abundant in the DNA and, until now, little is known about its mutagenicity and cytotoxicity (Fu et al., 2012; Kondo et al., 2010b).

Due to their carcinogenic and cytotoxic properties, the alkylating agents are considered dangerous to human health. However, some of compounds are commonly used as chemotherapeutic drugs in order to kill cancer cells. Although alkylating agents contribute to the onset of cancer, they also play a relevant role in the treatment of cancer (Fu et al., 2012). Most of the anticancer alkylating drugs used are monofunctional methylating agents (e.g. temozolomide [TMZ] and *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine [MNNG]) containing only one active chemical group for the alteration of a single site in DNA. The TMZ and MNNG are the S_N1-methylating agents because they alkylate not only nitrogen but also oxygen groups in DNA bases. Contrarily, compounds such as methyl methanesulfonate (MMS) belongs to S_N2-methylating agents because mainly alkylate nitrogen rings. Bifunctional alkylating agents (such as nitrogen mustards, e.g. cyclophosphamide and chlorambucil) and chloroethylating nitrosoureas (e.g. carmustine [BCNU], nimustine [ACNU] and fotemustine) contain two reactive sites and can interact with separate bases present in opposite DNA strands culminating in the formation of interstrand crosslinks (ICLs) in addition to monoadducts and intrastrand crosslinks (ligation between two nucleobases in the same DNA strand). The chloroethylating agents have the ability to add a chloroethyl group to nucleobases (Deans and West, 2011; Fu et al., 2012; Kondo et al., 2010b). Among all DNA lesions produced by these drugs ICLs are particularly toxic and lethal to cells because they inhibit the separation of the two strands of DNA and, consequently, block replication forks affecting replication and transcription processes (Deans and West, 2011). The mispair between O^6meG and thymine may also originate cytotoxic breaks when recognized by the MMR system.

Overall, chemotherapeutic alkylating compounds have the ability to change a range of biomolecules including DNA generating damages that may cause cell death by apoptosis, and therefore can be useful in the treatment of cancer (Fu et al., 2012).

The standard treatment applied in cancer patients is based on surgical resection followed by radiotherapy and chemotherapy in order to remove residual cancer cells. Over the years, chemotherapeutic alkylating agents (with methylating or chloroethylating properties) have received much attention in the treatment of several cancers such as melanoma, ovarian cancers and lymphomas. For instance, Dacarbazine was approved by FDA (with methylating properties) due to its efficiency for treatment of melanoma after surgical resection (Gerson, 2004).

Alkylating anticancer drugs are widely used in glioblastoma patients. The main reason for application in this type of tumors is due not only their ability to easily cross the blood-brain barrier but also their efficient absorption after administration. First, only nitrosoureas chemotherapeutics (such as CCNU and BCNU) were approved and applied for the therapy of glioblastomas. However, these agents only offered a small improvement in the quality of life of these patients (Rhee et al., 2009). To attempt to overcome this problem, more recently, TMZ was implemented as standard chemotherapeutic drug for this type of cancer because an enhancement in median survival of patients was observed. Some studies, mainly in glioma patients, also demonstrated that the addition of chemotherapy based on alkylating agents to radiotherapy prolongs the median of survival compared with patients subjects only a radiotherapy (Stupp et al., 2005). Therefore, the concomitant and adjuvant application of alkylating chemotherapeutic agents after surgical resection have been applied in some solid tumors and are a good strategy that provides survival benefits.

1.4. Mechanism of DNA repair

As above mentioned alkylating agents can promote the development of many kinds of DNA alkylated base lesions and normally, organisms respond by repairing the damage through to several pathways and, consequently, preventing genomic instability. The most important DNA repair systems that are involved in repair of alkylation damages include: mismatch repair (MMR), base excision repair (BER), nucleotide excision repair (NER), homologous recombination (HR) and non-homologous end joining (NHEJ) pathways (Hoeijmakers, 2001).The repair of DNA alkylating damages also can be executed by O^6-methylguanine-DNA methyltransferase repair protein (MGMT) through direct repair without removal of the damage base (Helleday et al., 2008). Collectively these pathways are major players in modulation of cell sensitivity to alkylating agents. It is thought that these repair pathways are interconnected and although they can be defined separately, they perform functions that overlap with each other (Knudsen et al., 2009). In order to maintain the genome stability, cells developed a network of pathways (which constitute the DNA damage response (DDR)) that allow the detection of DNA lesion, signals its presence and stimulate its repair. In mammalian cells, ATM (ataxia-telangiectasia mutated) and ATR (ataxia telangiectasia and Rad3-related protein) protein kinases (members of the phosphatidylinositol 3-kinase (PI3K) superfamily) are main components this signaling network. ATM are recruited and activated by DSBs, whereas ATR has been associated in response to diverse types of DNA lesions such as DSBs, crosslinks, monoadducts, SSBs as well as stalled replication forks (Cimprich and Cortez, 2008; Jackson and Bartek, 2009). The two direct targets of these kinases are the protein kinases CHK2 (checkpoint kinase 2) and CHK1 (checkpoint kinase 1), respectively (Bartek and Lukas, 2003). Consequently, these activation may promotes the cell cycle arrest allowing more time for repair the DNA before replicative process (Jackson and Bartek, 2009). In response, for example, to cytotoxic O^6-alkylG DNA adducts induced by alkylating agents occurs the ATR activation. In this case, ATR promotes the phosphorylation and activation of CHK1 and p53 that in turn trigger apoptotic pathways and cell cycle arrest. For instance, the S_N1 –type alkylating agents induced arrest in the G2/M phase of cell cycle and posteriorly apoptosis in the second cell cycle after treatment and these events were preceded by activation of ATR and phosphorylation of CHK1 and p53 (Yoshioka et al., 2006).

1.4.1. Direct damage reversal repair

The main mechanisms that support the repair of alkylated lesions comprise the O^6-methylguanine-DNA methyltransferase (MGMT) protein. MGMT operates in cells in order to inhibit the effects of chloroethylating and methylating agents and repairs O^6-alkylG lesions (Kaina et al., 2010). MGMT is described as DNA suicide enzyme that supports the elimination of the methyl or chloroethyl group linked in the O^6 position of guanine and, this group, is transferred to the cysteine residue present in the catalytic site of MGMT. This reaction is irreversible and culminates in inhibition of the protein that, posteriorly, undergoes ubiquitination and degradation in the proteasome (Figure 1.4) (Verbeek et al., 2008).

Therefore, the repair capacity of O^6-alkylG is mainly determined by the number of active MGMT molecules presents in the cells (Kaina et al., 2010). Lesions like O^6meG are considered highly mutagenic, and if MGMT is not enough to remove all lesions, during replication this lesion may interact with thymine rather than cytosine causing GC-AT transitions (Eker et al., 2009).

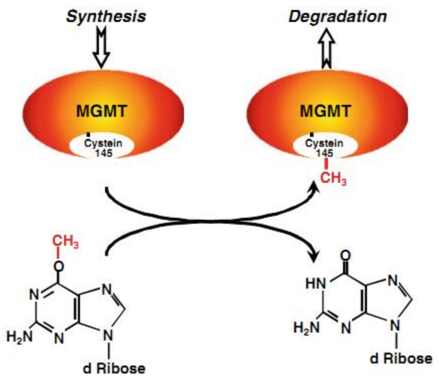


Figure 1.4 - Representation of the repair reaction by MGMT molecule (Adapted from (Kaina et al., 2010)).

However, for a long time it was not understood how this type of lesion, that does not stall DNA replication, can be highly cytotoxic. After some studies realized in cells defectives in MMR pathway it was observed that the cytotoxicity of the O^6-meG is mainly due to the recognition of mispairs by the MMR pathway (Levati et al., 1998; Liu et al., 1996; Quiros et al., 2010).

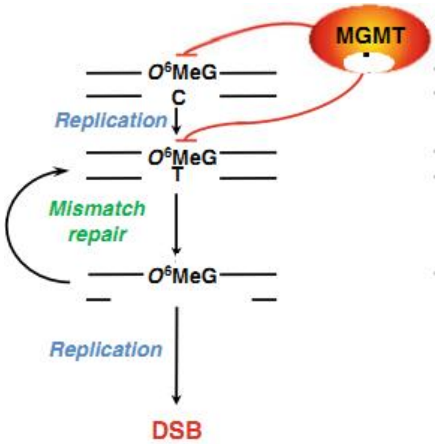


Figure 1.5 - Action mechanism of the MGMT system (Adapted of Kaina et al.,

This system promotes the elimination of the thymine existent in complementary strand creating a break and, consequently, getting the O^6MeG free to pair once more with another thymine in the next round of DNA replication. So, if O^6MeG remains in the strand, this leads to the repetition of the process triggering a futile cycle of repair. This cycle can culminate in chromosomal aberrations, cell cycle arrest or in the formation of DSBs that are powerful activators of apoptosis (Figure 1.5) (Bugni et al., 2009; Kaina et al., 2007). Due to formation of

DSBs caused by repetitive MMR processing, homologous recombination pathway (HR) can constitute another cellular mechanism against the cytotoxicity caused by O-alkyl adducts. In support of this, cells HR-deficient display an enhanced in sensitivity to O^6-alkylating agents (Roos et al., 2009). Besides of O^6meG lesions, MGMT can also promote the removal of primary adducts induced by chloroethylating agent (O^6-chloroethylguanine - O^6-ClethG). This way, the chloroethyl group attacks the O^6 position of guanine forming an optimal subtract for direct repair by MGMT (Drabløs et al., 2004).

MGMT have particular interest for cancer research due to its role in cancer prevention and cancer chemotherapeutic response. Therefore, MGMT promote the removal of O^6-alkylG adducts from DNA template induced by exogenous or endogenous compounds provides protection of normal cells. However, if levels of MGMT expression were elevated in cancer patients, this would lead to also the protection of tumor cells against chemotherapeutic agents since it was occurred the efficient elimination of O^6-alkylG lesions induced by these drugs.

It has been described that 50% of these cancers exhibit methylation of the CpG islands present in the MGMT promoter region leading to epigenetic silencing of MGMT gene and inhibition of ability to repair O-alkyl lesions from DNA (Lind et al., 2004). This methylation, which promotes the formation of inactive chromatin, occurs frequently in glioblastomas, head and neck carcinomas and has been observed in the early phases of carcinogenesis (Esteller et al., 2000). Thereby, the status of the MGMT promotor is recognized as a prognostic factor for therapy since absence of MGMT expression is considered a positive survival predictive marker in patients who are treated with alkylating chemotherapeutics (Liu and Gerson, 2006; Verbeek et al., 2008). In addition to promoter methylation there are other factors that influencing MGMT expression including transcription factors. The sequence of MGMT promoter contains many transcription binding sites such as glucocorticoid response element. Thus, induction of these sites results in enhances in MGMT expression, which are enough to cause more resistance to alkylating therapies. Dexamethasone, a synthetic glucocorticoid, is one example of a compound that capable to upregulate MGMT *in vitro* and reduces the efficiency of chemotherapy (Biswas et al., 1999). All of these facts support the view that chemical depletion of MGMT activity could be used to sensitize tumor cells to alkylating drugs. Therefore some strategies have been developed in order to enhance the response to these agents. One of the most potent inhibitors of MGMT activity is O^6-benzylguanine (O^6-BG) and has been applied in many studies realized *in vitro* and *in vivo* (Dolan et al., 1991; Kreklau et al., 1999; Rhines et al., 2000). The low molecular weight compound operates as a substrate for MGMT and this reaction leads to irreversible inhibition of the MGMT protein (Rabik et al., 2006). Due to its effectiveness, this compound have been used in combination with therapy based on alkylating agents and has shown promise in clinical trials with patients with glioblastoma (Quinn et al., 2009; Weingart et al., 2007). Unfortunately, the systematic application of O^6-BG and consequently, depletion of MGMT activity concomitantly with alkylating agents not only affects the tumor cells but also normal tissue cells can lead to myelosuppression (Fu et al., 2012). Therefore it is desirable to develop approaches for the specific targeting the MGMT-inhibiting agent to the tumor. One of the strategies, which have been done in patients with glioblastoma multiforme, is the local intracerebral administration of the inhibitor (Koch et al., 2007). To attenuate the myelosuppression, gene therapy may provide protection of bone marrow cells against the collateral toxicity of chemotherapeutic agents. Human MGMT cDNA enconding P140K or G156A are mutant versions of MGMT that are very resistant to inhibition by O^6-BG, that, ita transplanted into bone narrow will ensure high levels of expression of MGMT (Kaina et al., 2010; Verbeek et al., 2008). In conclusion, the modulations of MGMT concomitantly with alkylating agents together with strategies that protect stem cell are clearly approaches that could trigger major advances in therapy based on alkylating agents.

1.4.2. Mismatch repair

One of the DNA repair mechanisms that acts after replication is the mismatch repair (MMR) and it promotes the elimination of base-base mismatches which are made by DNA polymerases as well as insertion-deletion loops (IDLs) (Kunkel and Erie, 2005). In addition to the repair of the damages mentioned above, this system has a major impact on the level of damages caused by alkylating agents (such as TMZ) since can also mediates a O^6meG-induced cellular response. The O^6meG lesions that may mispair with thymine present in the complementary strand leads to the formation of O^6meG:T mismatches. These adducts are efficiently recognized by MMR system through to the MutSα heterodimer. Then, MMR, by the action of several proteins as will be described below, can promotes the removal of thymine from template creating a SSBs (Kaina et al., 2007; Martin et al., 2010). It is important noted that some studies referred that MMR system is only relevant in cases of treatment based on methylating agents contrary to chloroethylating agents and, this system acts after one cycle of replication (Kaina et al., 2010). However, this question needs to be clarified.

The MMR system targets the newly synthesized strand unlike NER and BER systems. In this pathway, two heterodimers are responsible for the recognition of the damage and initiation of repair: the hMutSα (which is constituted by MSH6 and MSH2 and recognizes base mismatches and small IDLs containing between one or two extrahelical nucleotides) and hMutSβ (a dimer of MSH3 and MSH2 responsible for the recognition of larger IDLs). The machinery of excision depends on the complex of MutSα, MutLα, exonuclease-1 (Exo1) and replication protein A (Kreklau et al., 1999). Therefore, through interaction between them the excision of the mismatch is stimulated and Exo-1 promotes the degradation of the strand in a 5'-3' direction. The RPA will stabilize the region of single-strand DNA. After ablation of the damage the activity of EXO-1 is inhibited by MutLα and the Pol δ/PCNA complex fills the break with complementary nucleotides and through the action of DNA ligase I the incision is closed (Jiricny, 2006) (Figure 1.6).

According to the current studies, proteins involved in mismatch repair have the ability to recognize DNA damage and signal to the cell cycle checkpoint directly, which can cause apoptosis and cell-cycle arrest. On the other hand, this mechanism can try unsuccessful remove the damage causing stall of replication forks. In this case, the SSBs will promote the recruitment of RPA and ATR-interacting protein (ATRIP). These proteins cause the activation of ATR kinase which in turn phosphorylates kinase CHK1. It has been described that cells deficient in hMutSα- and hMutLα fail to arrest cell-cycle when exposed to several types of DNA-damaging agents (Li, 2008).

It has been described that MMR-deficient cells are less sensitive to death induced by several types of chemicals (such as S_N1-type methylating agents) than cells with functional MMR system (Stojic et al., 2004). One explanation for this fact is that cells without MMR, in response to DNA damage, cannot phosphorylate p53 and p73 and this implicates ATR and ATM since these kinases are responsible for the phosphorylation of p53 and p73 (Li, 2008). It has been demonstrated that hMutSα and hMutLα are responsible for the recruitment of ATR-ATRIP to sites of damages, by physical interaction, in cells submitted to treatment with DNA damaging agents and, this way, MMR proteins are implicated in a signaling cascade that triggers cell cycle arrest and apoptosis (Yoshioka et al., 2006).

Several reports have helped to understand that MMR mechanism have significant impacts on the outcome of cancer therapeutic interventions.

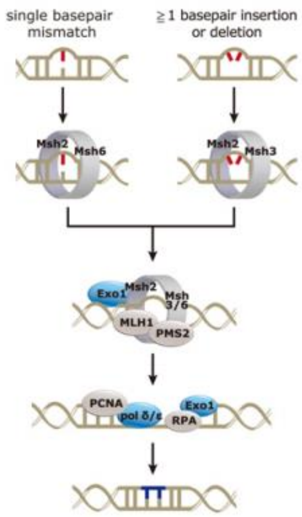


Figure 1.6 – Representation of mismatch repair pathway (Adapted from Genetex).

1.4.3. Base excision repair

Among the DNA repair pathways the base excision repair (BER) is one of the most highly conserved and occurs both in the nucleus and in mitochondrial DNA. This process identifies and repairs several damages including: SSBs and base lesions that arise due to oxidation (e.g. 8-hydroxyguanine (8-oxoG) or formamidopyrimidines), alkylation (N^7-alkylguanine, N^3-alkylguanine) or deamination and AP sites (Maynard et al., 2009). The first step of this process is the recognition of the damaged base by a DNA glycosylase which catalyzes the cleavage of the *N*-glycosidic bond (between the sugar and the base) forming an apurinic/apyrimidinic site (AP site). The DNA glycosylases is specific for the DNA lesion (e.g. in response to oxidative damage operates mutY homologue (MYH), Endonuclease III (NTH), human endonuclease VIII-like 1 (NEIL) and 8-oxoG-DNAglycosylase (OGG1); and in alkylating lesion are recognized by N-methylpurine-DNA glycosylase (MPG)) (Knudsen et al., 2009; Robertson et al., 2009). The cleavage of the AP site is carried out by the apurinic/apyrimidinic endonuclease (APE1) giving rise to 5'deoxyribose phosphate (dRP) and 3'OH ends. Then, the DNA polymerase β (Polβ) will add a complementary nucleotide to fill the gap. To finish the process, a DNA ligase seals the incision (Figure 1.7) (Hegde et al., 2008). BER can be sub-divided into two divergent pathways depending on the location (active or inactive regions of the genome) as well as nature of the lesion. When only one nucleotide is removed, this process occurs by short-patch BER (SP-BER), however, during long-patch BER (LP-BER) about 2-6 nucleotides are integrated at the damaged site (Maynard et al., 2009). Some targets of alkylating agents (such as temozolomide and carmustine) are the *N*-residues of purines and pyrimidines. The N-alkylpurines (such as N^7-alkylG, N^3alkylG and N^3-alkylA) are mainly repair through BER pathway and the DNA adducts are recognized by MPG glycosylase promoting its removal from DNA strand (Roos et al., 2009).

Several studies have demonstrated that this repair pathway is connected with cancer. It has been established that defects in proteins involved in BER pathway cause an increased risk of numerous types of cancer including colon cancer (Wilson and Bohr, 2007). Some lesions, like 3meA, are very mutagenic and toxic for normal cells and it is essential that this pathway is functional in order to avoid an accumulation of these toxic lesions leading to development of cancer. These defects, on the other hand, can be crucial for treatment of cancer. For example, cancer cells with defects in Polβ are more sensitive to MNNG and TMZ and consequently increasing the cytotoxic effect of alkylating chemotherapeutic agents (Sobol et al., 1996). Currently, some clinical are under development with the aim to potentiate the downregulation of BER or modulate enzymes involved in this pathway in order to increase alkylating drug sensitivity. So, small-molecule inhibitors of Pol β, inhibitors of APE activity and chemical modulation of poly (ADP-ribose) polymerase (PARP) are some examples of strategies applied in cancer cells (Bapat et al., 2010; Mégnin-Chanet et al., 2010; Wilson et al., 2010).

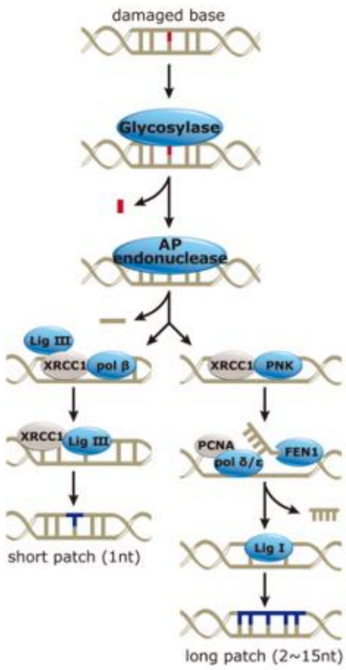


Figure 1.7 – Representation of base excision repair pathway (Adapted from Genetex).

1.4.4. Nucleotide Excision Repair

In the organism, the most versatile mechanism of DNA repair is the nucleotide excision repair (NER), which can identify and repair several DNA lesions. Cyclobutane pyrimidine dimers and (6-4)-photoproducts are lesions often created by ultraviolet light and that are processed by this pathway. Besides these damages, the NER pathway also contributes to the elimination of damage produced through the action of alkylating agents (e.g. interstrand crosslinks-ICLs) (Hanawalt, 2002; Kondo et al., 2010b). All these adducts are very cytotoxic for cells and change the normal structure of DNA affecting processes such as replication or transcription. NER can be categorized into two different pathways: global genomic repair (GGR, which processes injuries in the whole genome, including non-transcribed strands) and transcription coupled repair (TCR, which operates in the transcribed DNA strands). NER pathway comprises numerous steps such as: lesion recognition and structural distortion of the DNA (by XPC-HR23B-Cen2 complex); denaturation, nearly 30 nucleotides, of the strand around the damage (Transcription factor II human (TFIIH)); incision of the damaged (XPG in 3'end and ERCC1-XPF in 5' terminus); removal of lesion and the break is closed by the DNA polymerase and the ligation of the nick occurs by action of the DNA ligase III (Figure 1.8) (Nouspikel, 2009).

Like BER, also NER play an important role in treatment of cancer mainly if the patients receiving treatment based on the alkylating agents that promote the formation of ICLs. For example, several studies demonstrated that, patients with ERCC1 negative cancers that received adjuvant chemotherapy have a better prognosis and are more sensitive to alkylating agents (Olaussen et al., 2006).

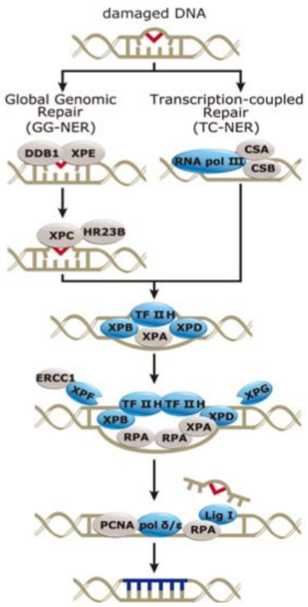


Figure 1.8 – Representation of nucleotide excision repair pathway (Adapted from Genetex).

1.4.5. Homologous recombination and non-homologous end-joining repair

The DNA double-strand break is one of the most lethal lesions in human cells that can provoke genetic instability or cell death. Owing to these consequences the cells developed two distinct pathways that may be used for its repair: homologous recombination (HR) and non-homologous end-joining (NHEJ) (Ohnishi et al., 2009). Despite of alkylating agents do not have the ability to induce directly DSBs, these type of lesion are identified in a final event after the treatment of cancer cells with methylating and chloroethylating agents (Kondo et al., 2010a; Kondo et al., 2009). The great difference between them is that HR uses DNA sequence homology to promote the DNA repair. HR requires the presence of an undamaged homologous DNA sequence (sister-chromatid) that serve as a template allowing repair the missing sequence at the DSB site. On the other hand, NHEJ is a simplest mechanism that also promotes the DSBs repair and, in this case, it observed a re-ligation of damaged DNA ends without needing a template (Dudáš and Chovanec, 2004). This mechanism is more important essentially for the removal of DSBs during the G1-phase of cell cycle because HR is not proficient owing to the absence of a homologous sequence. Contrary to HR system, NHEJ does not maintain the original genetic information because the break ends can be processed allowing the formation of appropriate substrates which, after, can be directly connected (Dudáš and Chovanec, 2004; Ohnishi et al., 2009).

1.5. Clinical chemotherapeutic agents – Temozolomide (TMZ) and Carmustine (BCNU)

As mentioned previously, due to their capacity to induce several damages to DNA some alkylating agents are frequently used in cancer therapy. Nonetheless, response and toxicity of these agents is strongly modulated not only by DNA repair pathways but also by the genetic variability present in several cancers.

BCNU, a chloroethylating agent, is an adjuvant agent very important in treatment of several cancers including brain tumors, lymphomas and melanomas. BCNU causes cytotoxic effects due to alkylation of O^6 position of guanine (O^6-chloroethylguanine - O^6-ClethG). This monoadduct is converted through to internal cyclization into N1-O^6-ethanoguanine (N1-O^6-ethanoG) that is capable to react with cytosine present in the complementary strand (N3-cytosine) leading to the formation of N1-guanine-N3-cytosine interstrand crosslinks (ICLs) (Cui et al., 2009; Verbeek et al., 2008). BCNU also induces other types of DNA lesions such as monoadducts derived of alkylation in N1 and N7 positions of guanine (that do not lead to ICLs) (Cui et al., 2009). DNA damages created by this agent involve complex repair pathways. The O^6-ClethG are initially repaired by MGMT. Otherwise, the nucleotide excision repair, Fanconi anemia (FA) repair and homologous recombination (HR) are examples of downstream mechanisms required for complete depletion of interstrand crosslinks. Due to their role on repair of the DNA lesions responsible to induction of cell death, the DNA repair pathways contribute to cancer cell resistance to BCNU (Drabløs et al., 2004; Kondo et al., 2010b; Verbeek et al., 2008).

Temozolomide (TMZ), another DNA alkylating anticancer agent, is frequently used to treat brain tumors including glioblastoma multiforme, gliomas and anaplastic astrocytomas as well as melanomas. The small molecular weight and lipophilic proprieties of TMZ are the main characteristics which makes this agent highly efficient in these types of cancers (Koukourakis et al., 2009). Recently, TMZ has been applied in patients with metastatic colorectal cancer and a partial tumor regression was observed (Shacham-Shmueli et al., 2011).

This agent does not require hepatic metabolism for its activation and is rapidly absorbed after oral administration. TMZ is a triazene compound that hydrolyses spontaneously transforming in the active metabolite MITC [5-(3-dimethyl-1-triazen-1-yl) imidazole-4-carboxamide]. MTIC rapidly reacts and release carbonium ions that specially alkylate DNA at N7 position of guanine (N^7meG – about 70%), as well as N3 position of adenine (N^3meA – about 9%) and O6 residues of guanine (O^6meG – about 6%) (Zhang et al., 2012).

Just as in the case of BCNU, the primary DNA lesion O^6-methylguanine (O^6-meG) induced by TMZ is also eliminated by the DNA repair protein MGMT. However, the toxicity of TMZ is also dependent on the mismatch repair system that acts after the first cell cycle of replication. This DNA repair mechanism is important for this class of compounds because promotes the generation of DSBs that trigger cell death by apoptosis (Quiros et al., 2010). For successful repair of N-alkylations, caused by TMZ, the activation of functional BER system is also necessary (Liu and Gerson, 2006).

Therefore, when applied alkylating agents, it becomes important an introduction of routine genetic analysis of patients tumors in order to develop individualized treatments which will allow better prognosis.

1.6. Natural compounds and chemoprevention

Due to the frequent exposure to environmental oxidanting and alkylating compounds, the diet and lifestyle have a great impact at the level of prevention of DNA damage and as a consequence of many diseases, such as cancer. Through epidemiological studies it has been possible to establish that a diet abundant in plant foods and some phytochemical components helps in the prevention of numerous cancers (chemoprevention), for instance, CRC (Liu, 2004; Pan et al., 2008; Ramos et al., 2008). Studies revealed the role of phytochemicals (mostly found in vegetables and fruits) in CRC. For example, quercetin, ursolic acid and luteolin showed to have anti-proliferative and pro-apoptotic effects in cell lines derived from CRC (Xavier et al., 2009). Thus, there is a growing interest in trying to isolate and identify specific compounds existent in the diet that may have effects on DNA protection and induction of DNA repair pathways. Overall, chemoprevention can be defined as a process in which natural or synthetic compounds are used to delay, reverse or prevent the growth tumor and may control its behavior through modulation of metabolic mechanisms and signaling pathways (Pan et al., 2008).

Phytochemicals are an example of bioactive compounds present in plants that can act as antioxidant by neutralizing free radicals and oxidants, can induce iron chelation, promote the stimulation of cellular defense systems, and regulate the gene expression in cell proliferation, cell differentiation. They can also induce cell-cycle arrest or apoptosis. Phytochemicals can be divided into: carotenoids, alkaloids, polyphenols, nitrogen-containing compounds and organosulfur compounds. Polyphenols can be sub-divided into 4 groups, according to the amount of phenol rings: flavonoids, lignans, stilbenes and phenolic acids. On the other hand, flavonoids also can be classified into 6 subgroups: flavonols, flavones, isoflavones, flavanones, anthocyanidins and flavanols (Liu, 2004).

The most common dietary polyphenols are the flavonoids, like luteolin, rutin, quercetin, curcumin and resveratrol, and phenolic acids, including rosmarinic acid. Flavonoids have the ability to act as metal chelators and as free radical scavengers. These compounds may inhibit not only molecules involved in the inflammatory processes that culminate in transformation, proliferation and initiation of carcinogenesis but also may suppress the final steps of carcinogenesis namely metastasis and angiogenesis (Aggarwal and Shishodia, 2006). For instance, resveratrol and curcumin (present in grapes and turmeric, respectively) have the ability to downregulate the expression of apoptosis suppressor proteins Bcl-2 and NF-kB, and increase caspases activity and Bax levels. Quercetin (which is found in citrus fruit and apples) for example upregulate proteins involved in apoptosis such as Bax and caspases but also proteins that intervene in cell death and DNA repair.

MGMT, as previously mentioned, has an important role in cancer prevention of normal cells against exogenous and endogenous carcinogens promoting the removal of initial O^6-alkyl adducts from DNA template. This protein is the first line of defense when are formed alkylating damage in DNA. Thus, it becomes highly desirable, in terms of chemoprevention, that natural compounds have the ability to enhance the expression of MGMT. Studies have reported that curcumin, resveratrol and silymarin enhance MGMT expression levels in cancer cell lines (Niture et al., 2006a). Another study showed that some medicinal plants extracts, such as holy basil (*Ocimum sanctum*), oregano (*Origanum majorana*) and winter cherry (*Withania somnifera*) increased protein expression of MGMT. Although to a smaller extent, plant extracts derived from gooseberry (*Emblica officinalis*) or spearmint (*Mentha viridis*) also promote the increase of MGMT levels (Niture et al., 2006b). In addition, as tumor cells exhibit several methylated genes, some constituents of the diet such as (2)-epigallocatechin 3-gallate (EGCG) and genistein have been demonstrated to have the ability to reactivate the *MGMT* gene epigenetically silenced. This effect is associated with the inhibition of DNA methyltransferases (DNMT) that results in the demethylation of GpC islands in the MGMT promoter (Fang et al., 2007). These results show a beneficial effect for cancer prevention because would provide the removal of alkylating damages introduced in DNA but, on the other hand, may decrease the effectiveness of cancer therapy since this protein would remove DNA damages if cancer patients are treated with alkylating chemotherapeutic agents. Chemopreventive agents have been demonstrated that may help improve the efficiency of cancer patients, when applied concomitantly with chemotherapy (Sarkar and Li, 2006). However, diet may also be used to improve the effects of chemotherapy with alkylating agents and, therefore, compounds that decrease MGMT activity or increase MMR repair should be chosen.

1.7. p53 – the tumor suppressor

The tumor suppressor p53 is encoded by the *TP53* gene and has a relevant role in cancer being referred to as the "guardian of the genome" due to the many functions it exercises to maintain cell homeostasis. It is recognized as a transcription factor involved in the regulation of many genes being usually present at low levels in normal cells. This protein is negatively regulated by MDM2, an E3 ubiquitin ligase, which targets p53 for ubiquitination allowing its elimination by the proteasome. p53 is activated in response to some stresses such as DNA damage (radiation, UV, chemotherapeutic drugs), hypoxia, heat shock and oncogene activation (Chari et al., 2009). In response to these stimuli, post-translational modifications occur in this molecule that will promote its translocation to the nucleus and binding to specific DNA sequences that are present in the promoters of target genes. Activation of p53 leads to improved DNA repair, cell-cycle arrest, stimulation of cell death by apoptosis or senescence (Chari et al., 2009). The induction of apoptosis by p53 occurs by direct interaction with components involved in mitochondrial pathway of apoptosis through to the inhibition of anti-apoptotic proteins (namely, Bcl-2 and Bcl-x) and activation of pro-apoptotic family proteins (including BAX, PUMA, FAS receptor, APAF-1 but also FasR) (Kaina et al., 2007; Vaseva and Moll, 2009). In response to DNA damage, there is the activation of some DDR proteins, namely ATM and ATR. CHK1 and CHK2, the targets of ATR and ATM respectively, phosphorylate p53 which reduces the degradation of p53 by MDM2. Activation of p53 induces for instance in the case of DNA repair GADD45α, XPC, OGG1 and DNA polymerase (Sengupta and Harris, 2005).

One of the main reasons for p53 to be one the most studied proteins is that about 50% of all cancers, including colorectal, have inactivating mutations of p53 (Lu and El-Deiry, 2009). It has been widely reported that tumors that retain a wild-type p53 gene sequence have better sensitivity to chemotherapy and radiotherapy (Chari et al., 2009). For instance in ovarian cancer patients, an association between resistance to cisplatin treatment and expression of mutant p53 gene was found (Shelling, 1997). Also, colorectal and gastric cancers that contain mutations in p53 sequence were less chemosensitive (Hamada et al., 1996). In contrast to previous data, a recent study demonstrated that melanoma cell lines with WTp53 were more tolerant to TMZ when compared with p53 mutant lines deficient in the MMR system (Naumann et al., 2009). Also, Blough and collaborators described that glioma cell lines without p53 are more susceptible to TMZ than cells with functionally wt p53 expression (Blough et al., 2011).

Therefore, more experiments are needed to clarify the interaction between the therapeutic agents and p53 protein in order to improve treatment. In particular it is necessary to clarify the involvement of p53 with regard to the use of alkylating agents.

1.8. Apoptosis

Apoptosis or programmed cell death is a fundamental cellular process of that plays a crucial role in cellular homeostasis (Qiao and Wong, 2009).

During the apoptotic process several morphological modifications occur in cells including: plasma membrane blebbing, formation of apoptotic bodies and nuclear chromatin condensation. In the early phase, after plasma membrane blebbing a “budding” process occur that consists in division of cell fragments into apoptotic bodies (surrounded by intact plasma membrane).

Apoptosis can be divided in two important pathways: the intrinsic pathway (also identified as the mitochondrial pathway) and extrinsic pathway (also designated as the death receptor pathway). The two pathways differ in some aspects. The intrinsic pathway is activated by several cellular stresses including DNA damage, ionizing radiation, hypoxia and ROS. Briefly, the cellular injury promotes the permeabilization of the outer mitochondrial membrane leading to the release of cytochrome c for cytoplasm. The cytochrome c binds to the Apaf-1 (apoptotic protease-activating factor-1) causing the activation of procaspase 9 creating the apoptosome. This complex then activates effector caspases including caspase 3 and 6. On the other hand, the extrinsic pathway is activated through ligand binding of death receptors (located in the plasma membrane). Tumor necrosis factor-α (TNF-α) and Fas-L are examples of death ligands and, its binding to the receptor promote the recruitment of death signal adaptor proteins (such as Fas-associated death domain (FADD)) and activation of procaspases 8 or 10. In turn, these procaspases activate effector caspases such as 3 and 9. Despite of the differences described above, it is possible noted that a crosslink exists between the two pathways (Qiao and Wong, 2009).

Defects in this cellular process confer cells a survival advantage leading to the malignant transformation. Therefore, deregulation of apoptotic pathways have been frequently linked to cancer initiation, progression as well as resistance to chemotherapy also in CRC. Cancer therapies are often based on induction of apoptosis in cancer cells and it has been amply recognized as a good therapeutic strategy for several cancers. In CRC, some alterations in the expression of proteins involved in apoptotic process have been described lead to chemoresistance. For instance, changes in caspase activation (which are cysteine proteases) including mutations in caspase 3, caspase 8 have been described. Frequently, alteration in expression levels of Bcl-2 family members, e.g. high expression of anti-apoptotic proteins (such as Bcl-2 and Bcl-X) and low levels of pro-apoptotic proteins (for example, Bax and Bak) have been established in CRC (Hector and Prehn, 2009).

A major inducer of cell death by apoptosis is DNA damage. Alkylating agents, such as BCNU and TMZ, are some examples of compounds that exert their cytotoxicity based on the induction of the DNA damage and induction of DDR, leading to cell cycle arrest and apoptosis. Apoptosis induced by these compounds is the consequence of blockage of the replication process by stalled replication forks and formation of DSBs. DSBs are detected by DDR signaling which signal downstream factors such as CHK1, CHK2 and p53. The transcriptional activation of pro-apoptotic proteins (including Bax and FasR) occurs. Several studies have shown that apoptosis caused by chemotherapeutic agents is due to induction of DNA damages. Methylating agents capable of inducing O^6meG lesions trigger apoptosis by the combined action of MGMT and MMR systems. If this lesion remains in the template, after two cycles of replication, leads to DSBs formation and induction both apoptotic pathways. On the other hand, the processing of *N*-alkylations may also trigger apoptosis if repair by BER system is deficient and these lesions can create AP sites and SSBs that induce blockage of DNA replication process resulting in DSBs (Roos and Kaina, 2013). In the case of chloroethylating agents, the induction of crosslinks are the mainly lesions that promote the blockage of replication and collapse of replication forks and consequently may trigger both apoptotic pathways (Roos and Kaina, 2006).

In this project the recently developed CoMeth assay will be used to confirm its applicability to the study of chemotherapeutic drugs (TMZ and BCNU) and the characterization of the involvement of MGMT, MMR and p53 in DNA damage produced and cell death induction.

2. RESEARCH OBJECTIVES

Alkylating agents are used in chemotherapeutic strategies due to their ability to induce DNA damage. These lesions can trigger cell cycle arrest and cell death. However, the use of alkylating anticancer agents is limited by resistance mainly due to mechanisms of DNA repair. MGMT and MMR are important players, and for instance, active MGMT and downregulation of MMR system benefits the cancer cells decreasing the induction cell death and, consequently, the responsiveness to alkylating anticancer agents. In other perspective, a normal function of DNA repair systems has a relevant role in the prevention of mutations that lead to carcinogenic process.

The comet assay is amply used for measure, for instance, oxidative DNA damages and evaluation of cellular effects induced by natural compounds. A simple reliable methodology to evaluate methylating DNA damage by the comet assay has been recently developed in our laboratory (CoMeth) using MGMT-inhibited and MMR-proficient cells. This tool allows explore the role of MGMT and MMR system in the response to alkylating agents.

In this context, the aims of this work are:

- Demonstrate the applicability of the CoMeth assay to measure alkylating DNA damages induced by methylating (TMZ) and chloroethylating agents (BCNU), namely, O^6meG and O^6ClethG, respectively, under MGMT inhibition with O^6-BG.

- Evaluate the role of MMR system for the DNA effects induced by two alkylating agents, TMZ and BCNU, on induction of DNA damage and cell death in colorectal cancer cells.

- Investigate how the expression of wt-p53 influences the sensitivity of colorectal cancer cells to alkylating drugs and the ability of the two alkylating agents to induce apoptosis.

- Evaluate the potential effects of some phytochemicals on expression levels of the repair enzyme MGMT.

3. MATERIAL AND METHODS

3.1. Cell lines, culture conditions and reagents

Caco-2 cell line (extracted from a human epithelial colorectal adenocarcinoma) is DNA MMR-proficient but harbors a G/A (Arg/His) mutation in codon 273 of the *p53* gene (Liu and Bodmer, 2006). This cell line was cultured as a monolayer culture in Dulbecco's modified Eagle's medium (DMEM)(Sigma-Aldrich, St. Louis, MO, USA), supplemented with 10% (v/v) fetal bovine serum (FBS) (Biochrom KG, Berlin, Germany) and 1% (v/v) antibiotics (100 U/mL penicillin and 100 µg/mL streptomycin)(Sigma-Aldrich, St. Louis, MO, USA).

HCT116 cell lines (derived from human colorectal carcinoma), p53-wild-type and p53 complete knockout for p53 cells (provided by Vogelstein) (Bunz et al., 1998). These cell lines contain homozygous mutation in *hMLH1* resulting in a nonfunctional protein, and consequently, in deficient DNA mismatch repair (MSI) (Table 3.1) (Goel et al., 2006). The cell lines were grown in RPMI-1640 medium (Sigma-Aldrich, St. Louis, MO, USA) and supplemented with 6% FBS (v/v), 1% (v/v) antibiotic/antimycotic solution and 0.1 mM pyruvate (Sigma-Aldrich, St. Louis, MO, USA). All cell lines were grown and maintained under an atmosphere containing 5% of carbon dioxide (CO_2) at 37°C. Cellular subcultures were performed when confluence reached values close to 80- 90%. Cells (Caco-2 and HCT116) were maintained and grown in 75 cm^2 and 25cm^2 tissue culture flasks (TPP) respectively. The medium was renewed 2 times per week. For subcultures and plating, the adherent cells were detached with trypsin solution 1% (Sigma-Aldrich, St. Louis, MO, USA) and, fresh medium was added in order to neutralize the trypsin and a cell suspension was obtained. Then, this suspension was loaded in a hematocytometer and the concentration of cells present in the suspension was estimated by the following formula: mean of cells in the four quadrants x dilution factor x 10^4 = number of cells per ml.

Table 3.1 – Mutations presents in Caco-2 and HCT116 colorectal cancer cell lines.

	Tumor suppressor p53 gene	**MMR system**
Caco-2 cells	Mutated	Wild-type
HCT116 cells	Wild-type	Mutated

Stock solutions of TMZ, BCNU and O^6-BG were prepared in dimethyl sulfoxide (DMSO) and aliquots were kept at -20°C. The three compounds were purchased from Sigma-Aldrich (St. Louis, MO, USA). The DMSO concentration that is placed in the culture medium in all conditions did not exceed 0.5% (v/v) and the controls were treated only with the vehicle (DMSO).

Ursolic acid (UA), Quercetin (Q), Curcumin (Curc), Silibinin (Sil) Resveratrol (Resv), Oleanolic acid (OA) and Rutin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Luteolin (L) was from Extrasynthese (Genay, France) and Rosmarinic acid (RA) from Fluka (St. Louis, MO, USA). All of stock solutions were dissolved in DMSO and aliquots were kept protected for light at -20°C.

Depending on the experiments, cells were plated in 12- (MTT and comet assay) or 6- (Western blot or nuclear condensation assay) multiwell culture plates with 1 or 2ml/well respectively, at a density of 0.1 x 10^6 (in the case of Caco-2 cells) and 0.6 x 10^5 (for the two isogenic HCT116 cell lines) cells/ml. For assays with application of alkylating agents, forty-eight hours after plating, the medium was discarded and fresh medium containing O^6-BG (100μM) was added 2h prior to alkylating treatment, for complete depletion of MGMT. Next, without changing the medium, the two alkylating agents, BCNU at 100μM and TMZ at 200μM, were added in order to promote DNA alkylating damage. As control, the cells were also incubated only with O^6-BG or DMSO (vehicle). On the other hand, for assays with natural compounds, forty-eight hours after plating, the medium is discarded and fresh medium with natural compounds were added.

For each method, the cells were harvested after specific periods of incubation, as will be described in the following sections.

3.2. Cell toxicity by MTT reduction assay

The MTT reduction assay was performed in order to select concentrations that cause significant inhibition of cell viability and, simultaneously, that induce alkylating DNA damage. This assay allowed us to determine the chemosensitivity of Caco-2 and HCT116 cells to TMZ, BCNU, O^6-BG, O^6-BG plus TMZ and O^6-BG plus BCNU treatments.

This colorimetric assay involves the conversion of the water soluble MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) in insoluble formazan, giving a purple color, allowing its spectrophotometric quantification. The bioconversion of MTT to formazan is performed by dehydrogenase enzymes found in metabolically active cells. This reduction involves the activity of intracellular oxidation-reduction systems, and consequently, the number of viable cells is directly proportional to the amount of formazan crystals produced (Sylvester, 2011).

After 48, 72 and 96h, 100 μL of MTT (5mg/mL, diluted in phosphate-buffered saline (PBS) solution and maintained in the dark and at 4°C) (Sigma-Aldrich, St. Louis, MO, USA) was added to each well and cells were incubated at 37°C, with 5% CO_2 during 1 hour, allowing the formation of formazan crystals. Then, the medium was removed from the wells and DMSO/ethanol solution (proportion 1:1) was then used to dissolve the formazan crystals. When all crystals were entirely dissolved, from each well 200µL of solution were taken and platted onto 96 wells and absorbance was measured with a microplate reader (SpectraMax Plus [384] Microplate Reader – Molecular Devices) at a wavelength of 570nm. As a blank, it was used only 200µL of DMSO/ethanol solution. The results were treated as a percentage of cell viability in relation to control (untreated cells, which was defined as 100% of cell viability). For that, the value correspondent to MTT reduction at the beginning of incubation (t = 0 h) was subtracted from all the experimental conditions, including the control. MTT negative values indicate cell death due to cytotoxicity while values between 0% and 100% indicate cell viability.

3.3. Nuclear condensation

In order to evaluate the effect of O^6-BG, TMZ, BCNU, O^6-BG plus TMZ and O^6-BG plus BCNU treatment on induction of apoptosis in Caco-2 cells and in two isogenic HCT116 cell lines the nuclear condensation assay was performed. After incubation time (48, 72 and 96h), the medium was collected to harvest dead cells and the attached cells were treated with trypsin solution during 5min. Trypsin was then neutralized with the respective harvest medium and collected to respective falcons that were then centrifuged at 2000rpm's to 10min and the pellet was ressuspended in 500µL of medium. Then, the cells were fixed with 4% paraformaldehyde (PFA) for approximately 20 minutes at room temperature and after this time they were washed once with PBS and centrifuged in the same conditions referred above. Finally, the solution inside the falcon was aspirated up to 500µL, the pellet was ressuspended in this solution and harvest to eppendorfs. Posteriorly, the cells were attached onto a polylysine-treated slide using a Shandon Cytospin at 500rpm for 5min. In the second step, polylysine slides were washed two times with PBS during 5min. In order to visualize apoptotic cells in the different conditions tested, the cells were stained with 20µL of Hoechst, which stains nuclear DNA, for 10min in the dark chamber. The dye was kept at 4°C in aliquots of 0.5 mg/mL (Sigma-Aldrich, St. Louis, MO, USA). This dye was diluted in PBS (1:100) so the final concentration was 5µg/mL. After this incubation time, 6µL of glycerol 50% was added in order to fix the coverslip to the slide. Under an inverted microscope equipped with a fluorescent light source (DAPI fluorescence filter) (Olympus IX71), the number of cells in each condition was determined. The percentage of apoptotic cells was calculated from the ratio between cells that exhibit nuclear condensation and total number of cells. For each condition, more than 500 cells were counted (Figure 3.1).

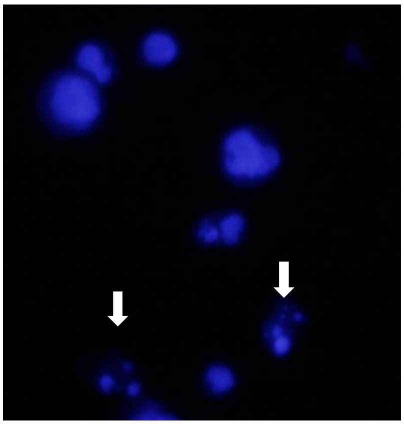


Figure 3.1 – Assessment of nuclear condensation and apoptotic bodies by Hoechst staining in Caco-2 cells exposed to BCNU treatment after 72h of incubation. Amplification 400 x. White arrow indicates cells with nuclear condensation.

3.4. DNA damage assessment by comet assay

In the present work we chose to evaluate the DNA damage induced by alkylating agents TMZ and BCNU under MGMT inhibition with O^6-BG through to the alkaline version of the single-cell gel electrophoresis assay (also known as CoMeth assay) as previously described (Collins, 2004).

The alkaline version is frequently recommended since it detects DNA damage, including SSBs, DSBs and alkali-labile sites (ALS) (Speit et al., 2004). This method for quantification of DNA damages has some advantages, such as: simple and fast; inexpensive; easy to execute in any tissue; allows the detection of multiple DNA damage (as referred above); allows evaluation of DNA lesions in individual cell; may be used in cases of low levels of DNA damage due to a high sensitivity; only requires a small quantity of cells per sample and can be applied in proliferative or non-proliferative cells (Hartmann et al., 2003).

To perform the comet assay, after 1, 48 and 72h of incubation of Caco-2 and HCT116 cells with BCNU (100µM) and TMZ (200µM), with or without O^6-BG (100µM), cells were washed in PBS solution, trypsinized and ressuspended in fresh medium. From each well was removed, to respective eppendorf, the volume required to obtain approximately 2 x 10^4 cells. This volume was centrifuged (at 0.6 rpm for 2min) and the resulting pellet was ressuspended in low melting point agarose (LMP, 0.5%). The mix was then placed on a slide, which was pre-coated with normal melting point agarose (NMP, 1%). After 10min at 4°C in order to solidify the agarose, the slides were placed in lysis solution (Annexes) during at least 1h at 4°C. This solution breaks the membranes and removes cellular contents, essentially histones, leading to the appearance of nucleoids of supercoiled DNA. After lysis, the slides were immersed in electrophoresis buffer (Annexes) at 4°C during 40 minutes under alkaline conditions (pH > 13) with the aim to occur DNA unwinding. Then, the slides were submitted to electrophoresis during 20min at 4°C, 21 V (0.8 V/cm) and 300mA. So, in this step the DNA that contains breaks migrates toward the anode leading to formation of the structures resembling a comet, with a head (that contains the intact DNA) and a tail (DNA loops that contains breaks). Afterwards, the slides are washed with water and dehydrated with absolute ethanol during 5min. Each sample was stained with specific nucleic acid fluorescent die (SYBRGold) during 30min at 4°C. After drying the slides were visualized on a fluorescence microscope (Leica, DM5000b + CTR5000 + ebq100) equipped with GFP filter. SYBR Gold was purchased from Invitrogen Molecular probes (Oregon, USA) and the aliquots were diluted in PBS 1X and maintained at 4°C (Figure 3.2).

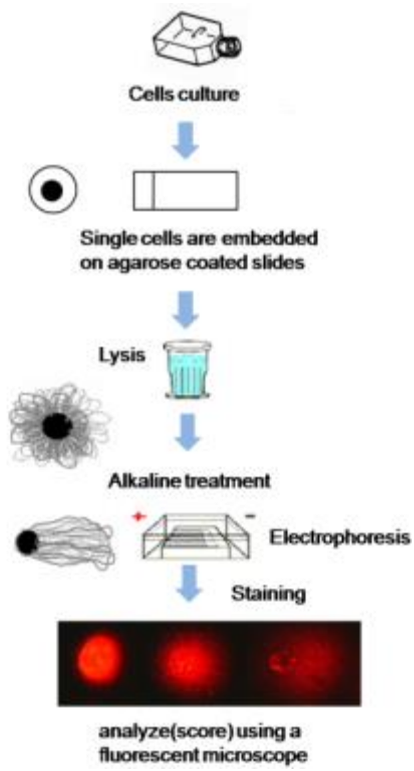


Figure 3.2 – Representative scheme of general steps involved in standard comet assay (Adapted from (Ramos et al., 2011)).

For definition and using the Comet IV analysis system (Perceptive Instruments Ltd, Haverhill, UK), approximately 100 cells are analysed per sample. Through this program it was possible to calculate the percentage of DNA in tail. Thereby, for every single cell this program measure intensity of fluorescence in the comet tail and head and attributes a percentage of DNA present in tail.

3.5. Protein extraction and Western Blot analysis

The effect of incubation with natural compounds (UA, Q, Curc, Sil, Resv, OA, R, L, RA) on MGMT protein expression, involved in the DNA repair of alkylating damage, was evaluated by Western blot after 48h.

After 48h of incubation, the cells were washed with phosphate-buffered saline (PBS) solution, trypsinized and medium was added for neutralizing the trypsin. The medium of each condition was collected to the respective falcon, the samples were centrifuged at 2000 rpm for 10min at 4°C. The cells were ressuspended in respective medium and harvest to individual eppendorfs. The cells were then lysed using ice-cold RIPA buffer (1% NP-40 in 150mM NaCl, 50mM Tris-HCl (pH 7.5), 2mM EDTA) supplemented with 20mM of sodium fluoride (NaF), 1mM phenylmethylsulfonyl fluoride (PMSF), 20 mM of Sodium orthovanadate (Na_3VO_4), and protease inhibitor cocktail (Roche, Mannheim, Germany) and samples centrifugated at 10 000 g (4° C) for 10 min. The soluble protein concentration was determined using Bio-Rad DC protein assay (Bio-Rad Laboratories, Inc., Hercules, CA, USA). The protein concentration of each sample was determined using a calibration curve with a standard protein solution, a bovine serum albumin (BSA). Posteriorly, for each condition 20μg of protein was separated through to 12% sodium dodecyl sulphate (SDS)-polyacrylamide gel. Protein band was transferred to Hybond-P polyvinylidene difluoride (PVDF) membranes (GE Healthcare). The membranes were blocked in PBS with 0.05% Tween-20 (TPBS) with 5% (w/v) non-fat dry milk, at least 2 h at room temperature and washed three times in TPBS solution. The membranes were then incubated overnight (4°C) with primary antibody (Table 3.2). Membranes were placed in secondary antibody (containing IgG horseradish peroxidase) during 1 h at room temperature, following three TPBS washes. Immunoreactive bands were detected through Immobilon solutions (Millipore, Billerica, MA, USA) and acquired using the chemiluminescence detection system (Chemi Doc XRS; Bio-Rad Laboratories, Inc.). As loading control B-actin antibody was used. The Quantity One software was used to quantify the area intensity of the bands. The results were expressed as relative percentage of control (β-actin). Control condition was considered to correspond to 100% of expression.

Table 3.2 – Antibodies and respective secondary antibodies used in western blot assay.

Primary Antibody	Dilution	Secundary antibody	Source
MGMT	1:500	Mouse	Santa Cruz Biotechnology (Santa Cruz, CA, USA)
MLH1	1:1000	Rabbit	Santa Cruz Biotechnology (Santa Cruz, CA, USA)
MPG	1:2000	Mouse	Abcam (Cambridge, UK)
APE-1	1:2000	Mouse	Abcam (Cambridge, UK)
β-actin	1:2000	Mouse	Sigma-Aldrich, St. Louis, MO, USA

3.6. Statistical analysis

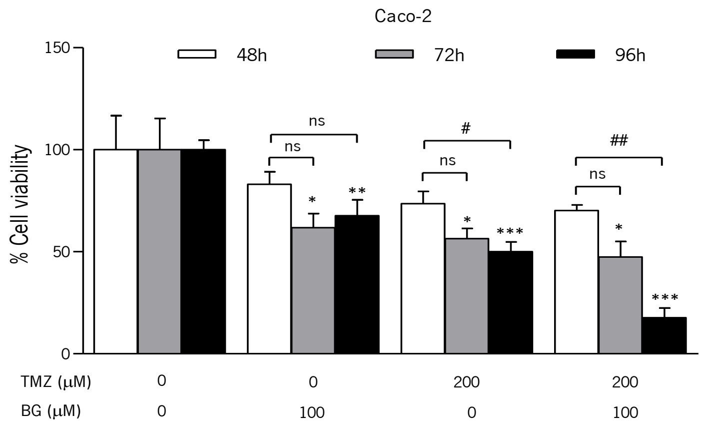
All experiments were performed at least three times and treated as means ± SEM. The results were evaluated statistically using the Student's *t*-test. Statistically significant differences between cells with or without pre-incubation with O^6-BG were considered when p-values ≤0.05 (* p ≤ 0.05; ** p ≤ 0.01;***p ≤ 0.001). The One-way ANOVA test followed by Tukey's Multiple comparison test was used to analyse differences in percentage of DNA damage as function of the time after treatment with or without O^6-BG pre-incubation. Significant differences were considered when p-values ≤0.05 (# p ≤ 0.05; ## p ≤ 0.01; ### p ≤ 0.001). All the statistical analyses were performed through to the GraphPad Prism 5.0 software (San Diego, CA, USA).

4. RESULTS

4.1 Effects of TMZ, BCNU and O^6-BG on cell viability

In order to define an adequate concentration of TMZ and BCNU agents, that are able to induce 50% of toxicity and cause DNA damages on Caco-2 cell line, a MTT assay was performed using several concentrations of the two alkylating agents in study (range between 1µM - 200µM) (Annexes). This experiment allowed us to choose the concentration of 200 µM to TMZ and 100µM to BCNU. So, for all experiments of this work were used the concentrations mentioned above. In order to evaluate the effect of pre-incubation with or without O^6-BG followed TMZ or BCNU treatment on cellular viability the MTT assay was performed (Figure 4.1-A and B). The defined time of incubation and the concentration of O^6-BG that significantly inhibited the MGMT, 100µM during 2h, have been defined in previous studies (Ramos et al., 2013).

(A)



(B)

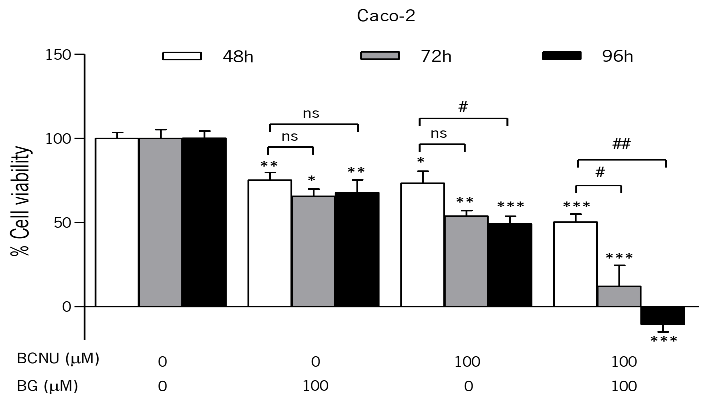


Figure 4.1 – Effect on cellular viability of **(A)** TMZ (200µM), O^6-BG (100µM), and O^6-BG plus TMZ **(B)** and BCNU (100µM), O^6-BG (100µM), and O^6-BG plus BCNU treatment on Caco-2 cell line, by MTT assay. The cells were pre-incubated during 2h with or without O^6-BG (100µM) before TMZ (200µM) or BCNU (100µM) treatment for 48, 72 and 96h. The percentage of cell viability was calculated by the absorbance relative to the value detected for the control cells (untreated cells), that was defined as 100% of cell viability. For each condition were performed three independent experiments and data are expressed as mean ± SEM. Significant differences were found, by Student's *t*-test, when compared with respective control (* P<0.05; ** P < 0.01; *** P < 0.001). Through One-way ANOVA significant differences over time of incubation (# P<0.05) were found.

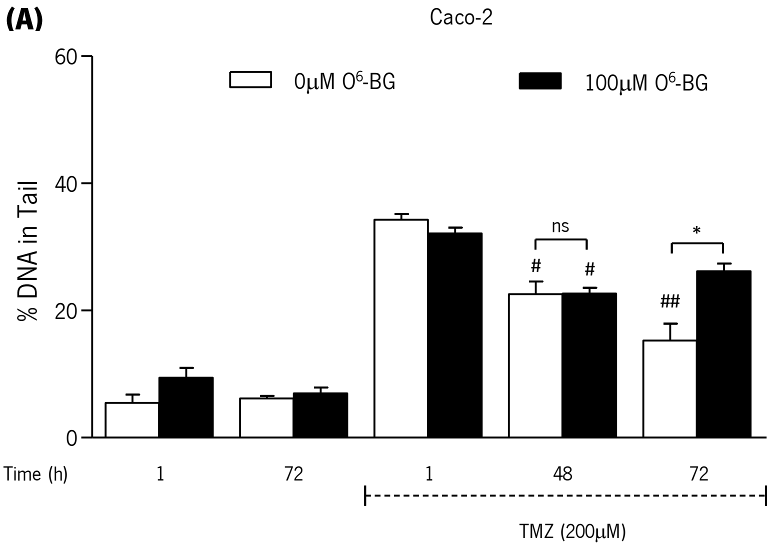
The results showed that (Figure 4.1 - A), when the cells were pre-incubated with the MGMT inhibitor, O^6-BG, followed by exposure of TMZ, there is a more pronounced decrease of viable cells compared to untreated cells. It was also observed that cells only treated with methylating agent TMZ exhibited a decrease of number of viable cells when compared with the respective control with reached values close to 50% of cell viability. Significant differences in cell viability were found after 72h of TMZ treatment with or without pre-incubation with O^6-BG. Relatively to BCNU treatment it was verified that cells treated with O^6-BG or BCNU for 48, 72 and 96h showed a significant decrease of cell viability when compared with the respective control (Figure 4.1 – B). The pre-incubation with O^6-BG followed by BCNU treatment decreased cell viability more drastically compared than the isolated drugs, mainly after 72 and 96h. At 96h, negative values were obtained in cells with O^6-BG plus BCNU treatment and this indicates that the induction of cell death occurred.

Therefore, for both alkylating drugs, a significantly higher inhibition of cell viability was observed in the presence of the MGMT inhibitor – O^6-BG potentiating BCNU and TMZ cytotoxicity.

4.2. Genotoxic effects of TMZ, BCNU and O^6-BG in Caco-2 cell line

We initially hypothesized that the comet assay could be applied to evaluate the levels of O^6-meG and O^6-ClethG lesions in DNA induced by TMZ and BCNU, respectively, in Caco-2 cells a MMR-proficient cell line. To test this hypothesis, Caco-2 cells were used and O^6-BG was used as an inhibitor of MGMT. This cells were subjected to treatment with TMZ (200μM) or BCNU (100μM) in the presence or absence of the MGMT inhibitor – O^6-BG (100 μM) at different incubation times (1, 48 and 72h). The parameter "percentage of DNA in tail" was used to evaluate the DNA damage induced (Collins and Azqueta, 2012).

After 1h of incubation with TMZ, an increase in DNA damage was observed with or without O^6-BG when compared with control cells (only DMSO) (Figure 4.2 - A).



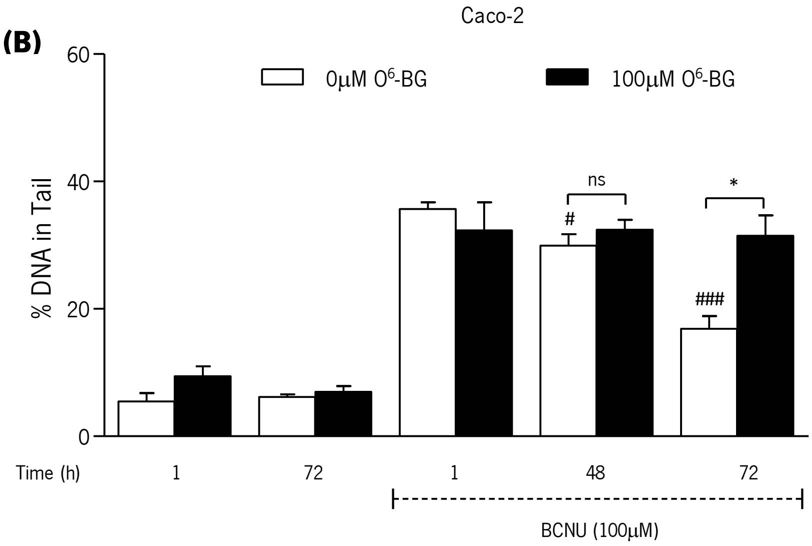


Figure 4.2 - Effect on DNA damage induced by **(A)** TMZ and **(B)** BCNU in the absence or presence of O^6-BG, evaluated by the comet assay, on Caco-2 cells. The cells were pre-incubated, during 2 hours, with or without O^6-BG (100µM) before TMZ (200µM) or BCNU (100µM) treatment for 1, 48 and 72h. For each condition were performed three independent experiments and data are expressed as mean ± SEM. Significant differences (*P < 0.05), determined by Student *t*-test, were found between cells with and without pre-treatment with O^6-BG at 72h. One-way ANOVA (followed by Tukey's Multiple comparison test) showed statistically significant differences over time between cells with or without pre-treatment with O^6-BG (# P<0.05; ## P<0.01; ### P< 0.001), when compared with 1h of treatment with or without O^6-BG, respectively.

It is also possible to observe that when Caco-2 cells were pre-incubated with O^6-BG followed TMZ treatment DNA damages remained high over time. However, the same pattern cannot be seen for cells treated only with TMZ because DNA damage decreased significantly over time. After 72h, a significant difference in DNA damage between cells without or with O^6-BG pre-treatment was detected. In both untreated cells and cells incubated with O^6-BG alone the DNA damages remained low during the complete experiment.

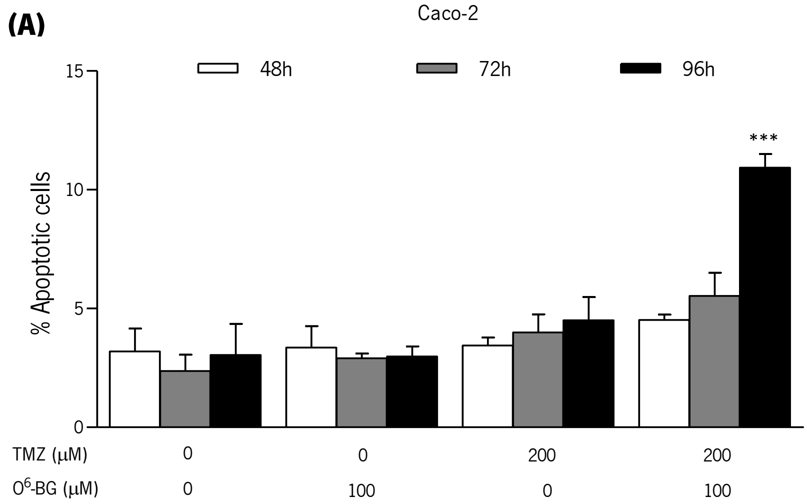
The chloroethylating agent, BCNU, seemed to have a similar effect to TMZ on Caco-2 cell line. As demonstrated in Figure 4.2 - B, after 1h of BCNU incubation, it was observed that BCNU significantly induced DNA damages with or without O^6-BG pre-incubation. During all time of the experiment, in the presence of O^6-BG, the DNA damage remained significantly high. On the other hand, over time a progressive and significant decrease in DNA damages was observed when cells were incubated with the alkylating agent only and a significant difference was found compared with cells pre-treated with O^6-BG after 72h of incubation. As expected, the results did not show any effects on DNA damages either in untreated cells (control cells) or in cells pre-treated with O^6-BG only.

Taken together, our results show a strong indication that O^6meG and O^6ClethG may be detected simply and quickly through the use of comet assay and, that, the presence of O^6-BG increased the genotoxicity of the chemotherapeutic drugs.

4.3. Effects of TMZ, BCNU and O^6-BG on cell death in Caco-2 cells

As mentioned above, both methylating and chloroethylating agents induced a progressive increase in DNA damage when cells were previously incubated with the inhibitor of MGMT protein, O^6-BG. Taken these results into account it was decided to explore if this DNA damage triggered cell death by apoptosis, as detected by the nuclear condensation assay.

The results obtained showed that Caco-2 cells pre-treated with the O^6-BG followed by TMZ treatment, showed a significant increase in the percentage of apoptotic cells at 96h of incubation. This result is in agreement with the results obtained for DNA damage at 72h, when cells were also pre-treated with O^6-BG before TMZ treatment. On the other hand, Caco-2 cells treated either with TMZ (200µM) or O^6-BG (100µM) alone did not show a significant enhancement in the number of apoptotic cells during entire time of the experiment (Figure 4.3 – A). As expected, control cells did not display any changes on the percentage of apoptotic cells over time of experiment.



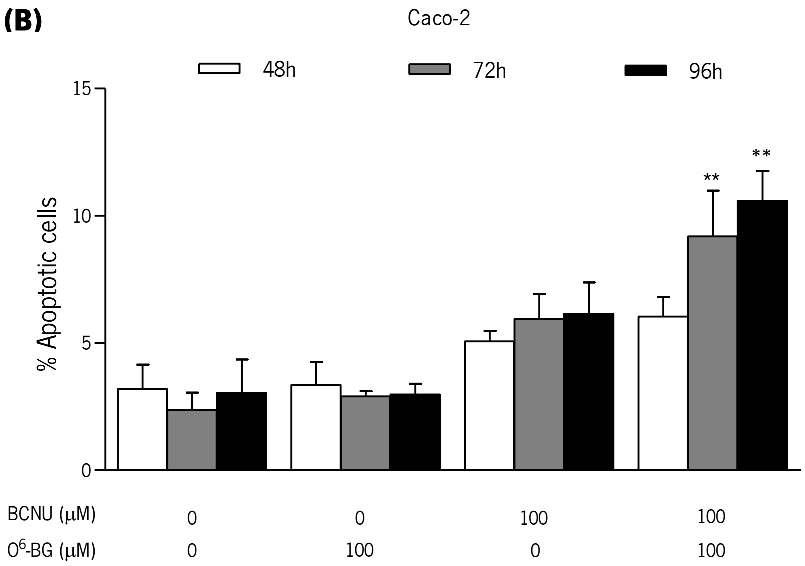


Figure 4.3 – Effect on induction of apoptosis, in Caco-2 cells, after incubation with **(A)** TMZ (200μM), O6-BG (100 μM) or O6-BG plus TMZ and with **(B)** BCNU (100μM), O6-BG (100 μM) or O6-BG plus BCNU. Apoptosis was evaluated through nuclear condensation assay after 48, 72 and 96h of treatment. The percentage of apoptotic cells were calculated through to the ratio between cells in nuclear condensation and the total number of cells. For each condition were performed three independent experiments and data are expressed as mean ± SEM. Significant differences were found, by One-way ANOVA followed by Dunnett's Multiple comparison test, when compared with respective control (untreated cells) (** $P < 0.01$; *** $P < 0.001$).

A similar behaviour was observed when cells were incubated with BCNU or O6-BG agents alone since an increase in the percentage of apoptotic cells during 96h of treatment did not occur (Figure 4.3 – B). When cells were pre-incubated with O6-BG followed with BCNU treatment, it was possible detect early (only after 72h) a statistical significant differences in percentage of apoptotic cells compared with respective control, since a pronounced increase occurred in this condition (about 5%). Over time control cells did not show any significant change in percentage of apoptosis.

4.4. Role of DNA mismatch repair on induction of DNA damages and apoptosis by alkylating agents

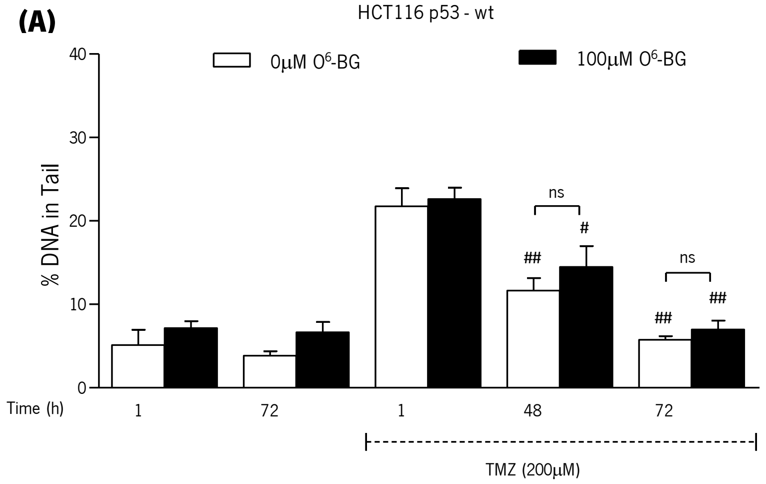
In this part of work, we try characterize the contribution of MMR DNA repair system for the recognition of O^6meG and O^6ClethG, initial lesions caused by TMZ and BCNU respectively, and formation of DNA strand breaks. For that, we used a colon cell line HCT116 that contains a mutation in *hMLH1* and consequently has a defective MMR system (Goel et al., 2006). Comparison with the results obtained with the MMR proficient Caco-2 cells will demonstrate the MMR dependence.

As shown in Figure 4.4 - A, after 1h of incubation with TMZ only or O^6-BG plus TMZ treatment significant DNA damages were detected by comet assay in HCT116 cells. However, at 72h for both situations (TMZ and O^6-BG plus TMZ), a significant reduction in percentage of DNA in tail occurred, i.e., the damages were almost entirely repaired. It is verified that at 72h, the amount of DNA damages was equivalent to the control values. Non-significant differences were found between cells with or without O^6-BG treatment followed by TMZ agent incubation.

The results demonstrate that, MMR deficient cells, even when the O^6meG lesions are not repaired due to MGMT inhibition, do not have the capacity to detect the alkylating DNA damage and promote strand breaks formation. In MMR-deficient cells, MGMT inhibition is not sufficient to cause SBs in DNA after methylating agent treatment.

As shown in Figure 4.4 – B, the DNA lesions induced by BCNU after 1h of incubation were elevated independently of whether the cells were pre-incubated with O^6-BG or not. It can be seen that, when MMR-deficient cells were pre-incubated with O^6-BG and then treated with BCNU the DNA damage (strand breaks) remained significantly high for 72h. On the other hand, the effects of BCNU treatment, without O^6-BG, on DNA damage decreased significantly over time. Consequently, at 48h cells exhibited significantly higher levels of DNA damages comparatively with cells without MGMT inhibitor. Over time of incubation, both untreated cells and cells incubated only with O^6-BG did not show significant variations in percentage of DNA damage.

(A)



(B)

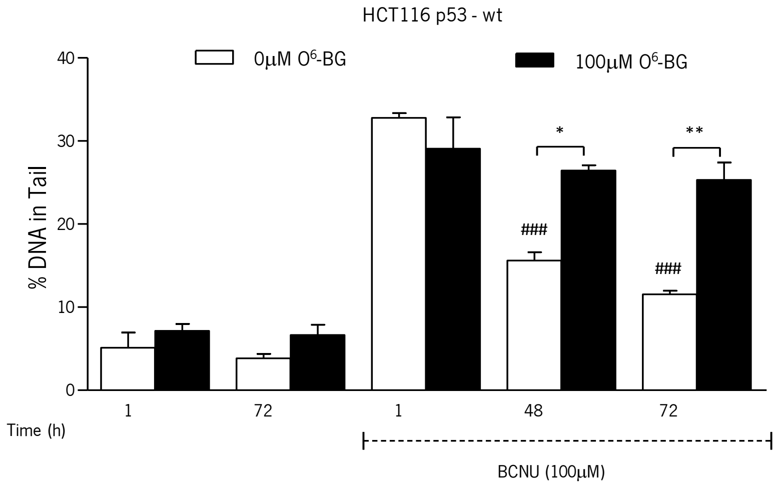


Figure 4.4 - Effect on DNA damage induced by **(A)** TMZ and **(B)** BCNU in the presence or absence of O6-BG evaluated by the comet assay on HCT116 p53-wt cell line. The cells were pre-incubated, during 2 hours, with or without O6-BG (100μM) before TMZ (200μM) or BCNU (100μM) treatment for 1, 48 and 72h. For each condition were performed three independent experiments and data are expressed as mean ± SEM. Significant differences (*P < 0.05; ** P < 0.01), determined by Student *t*-test, were found between cells with and without pre-treatment with O6-BG at 48h and 72h. One-way ANOVA followed by Tukey's Multiple comparison test, showed statistically significant differences over time between cells without pre-treatment with O6-BG (## P<0.01; ### P< 0.001), when compared with 1h of treatment.

Afterwards, to assess whether the O6meG and O6ClethG lesions that are converted during replication into SBs (that are detected by the comet assay) trigger apoptotic cell death we performed the nuclear condensation assay.

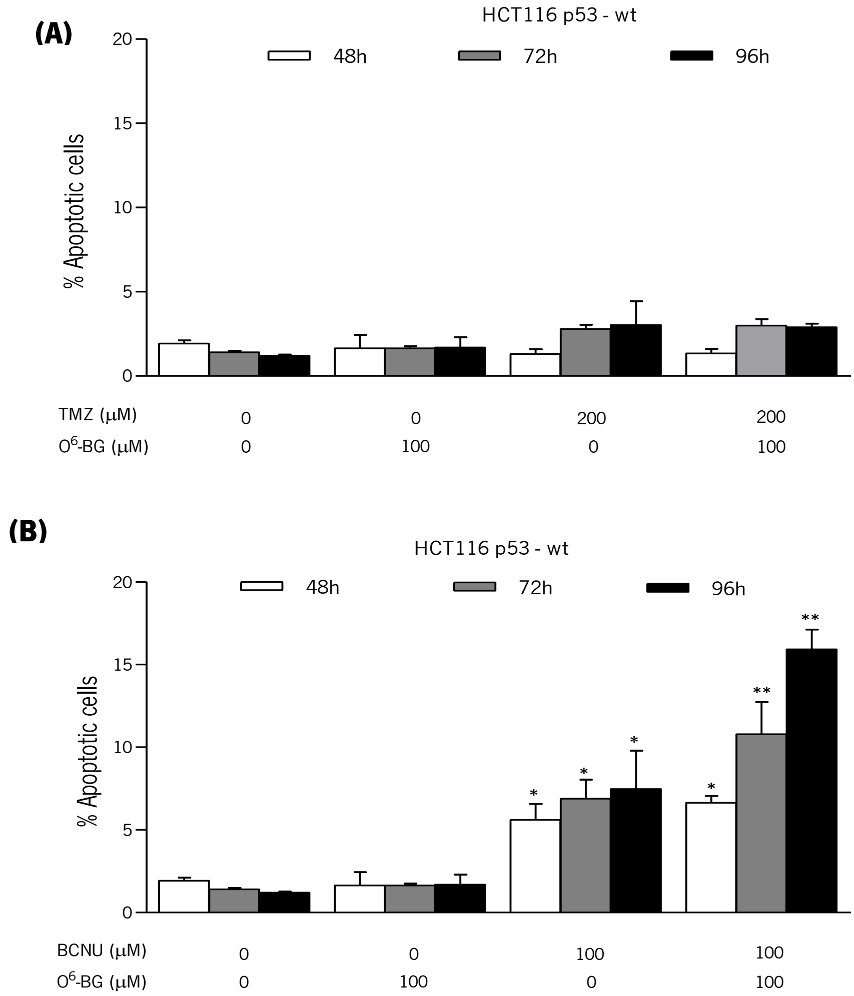


Figure 4.5 – Effect on induction of apoptosis, in HCT116 p53-wt cell line, after incubation with **(A)** TMZ (200µM), O6-BG (100 µM) or O6-BG plus TMZ and with **(B)** BCNU (100µM), O6-BG (100 µM) or O6-BG plus BCNU. Apoptosis was evaluated through nuclear condensation assay after 48, 72 and 96h of treatment. For each condition were performed three independent experiments and data are expressed as mean ± SEM. Significant differences were found, by One-way ANOVA followed by Dunnett's Multiple comparison test, when compared with respective control (untreated cells) (* P<0.05; ** P < 0.01).

As expected, the results obtained for cell death are in agreement with those described for comet assay in case of persistent DNA breaks. Therefore, neither O6-BG (100µM) nor TMZ (200µM) alone or in combination caused a significant enhanced of the percentage of apoptotic cells in this defective MMR cell line over the time (Figure 4.5 – A). Taken together, our results of TMZ demonstrated that the presence of functional MMR system is required for recognition and removal of the lesions leading to formation of breaks in DNA (that are detected by comet assay) that trigger cell death by apoptosis.

Conversely, in the case of BCNU treatment it was observed that, BCNU only as well as O^6-BG plus BCNU induced a significant increase of apoptosis compared with respective control cells (Figure 4.5 – B). However, more pronounced levels of apoptotic cell death were obtained only in the presence of MGMT inhibitor. Control cells and cells only incubated with O^6-BG did not show a significant variation in percentage of apoptotic cells. So, our results suggest that apoptosis induced by BCNU is mostly due to initial MGMT inhibition and may occur independently of the MMR system.

4.5. Role of p53 tumor suppressor gene on induction of DNA damages and apoptosis by alkylating agents

It is accepted that the levels of the tumor suppressor p53 gene plays a relevant role in several steps of the carcinogenic process as well as in the prognosis and response of cancer cells to chemotherapy (Ferreira et al., 1999).

Therefore, in this part of work, we intended to explore the involvement of the p53 tumor suppressor on DNA damage and induction of cell death by apoptosis in colorectal cancer cells treated with TMZ or BCNU. For this, we used for comparison two isogenic HCT116 colon carcinoma cells showing different status of p53: cells with p53 wild type (p53-wt) and cells knockout for p53 tumor suppressor (p53-null).

The results obtained for HCT116 p53-null cells (Figure 4.6 – A) demonstrated that the initial damages (after 1h of incubation) induced by TMZ or O^6-BG plus TMZ decreased progressively during the 72h of experiment. In addition, it is observed that after 72h the values of percentage of DNA in tail, in both conditions (TMZ or O^6-BG plus TMZ), were analogous to control values. The values obtained for control cells and cells pre-treated with O^6-BG alone remained low.

Through these results obtained with p53-null cell line, it can be suggested that the effects of methylating agents are MMR-dependent and, when MMR system is not functional, the functional status of p53 has no effect on extent of DNA damage induced by alkylating drugs to these cells.

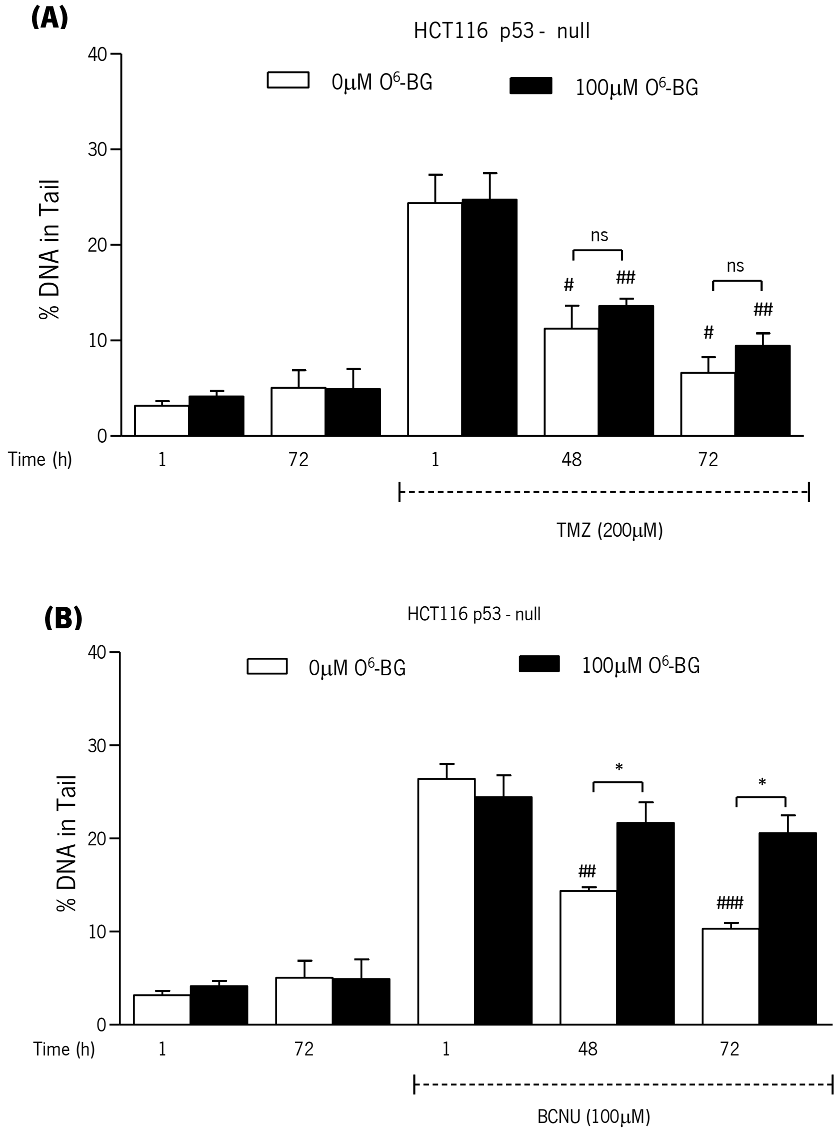


Figure 4.6 - Effect on DNA damage induced by **(A)** TMZ and **(B)** BCNU in the presence or absence of O^6-BG was evaluated by the comet assay on HCT116 p53-null cells. The cells were pre-incubated, during 2 hours, with or without O^6-BG (100μM) before TMZ (200μM) or BCNU (100μM) treatment for 1, 48 and 72h. For each condition were performed three independent experiments and data are expressed as mean ± SEM. Significant differences (*P < 0.05), determined by Student *t*-test, were found between cells with and without pre-treatment with O^6-BG at 48h and 72h. One-way ANOVA followed by Tukey's Multiple comparison test, showed statistically significant differences over time between cells without pre-treatment with O^6-BG (# P < 0.05; ## P<0.01; ### P< 0.001) compared with 1h of treatment.

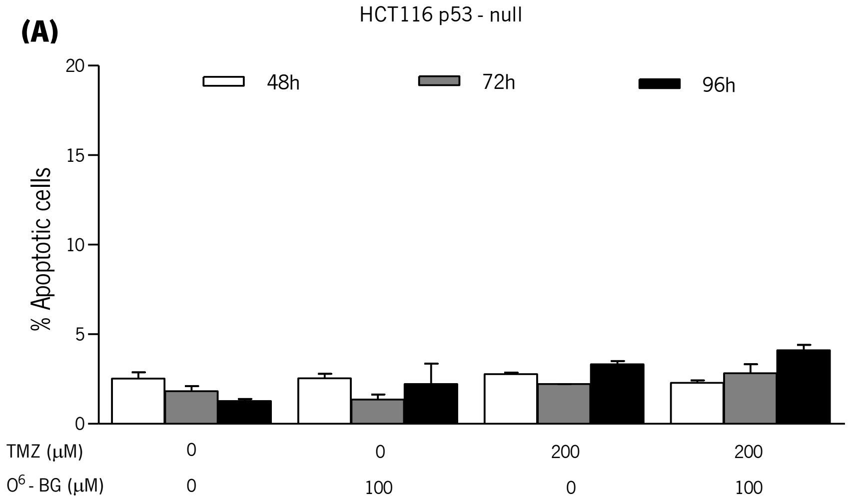
At 1h of incubation, also in this cell line, treatment with BCNU (Figure 4.6 – B), increased significantly levels of DNA damage regardless of the presence of O^6-BG. In the case of cells that were pre-incubated with MGMT inhibitor followed BCNU treatment, the DNA damages detected by comet assay remained elevated along the experiment. Contrarily, in cells treated with BCNU only, we found that, at 72h of experiment, the percentage of DNA damage induced by BCNU was significantly decreased and, significant differences were found compared with cells pre-incubated with O^6-BG. No significant alterations in percentage of DNA damages were detectable by comet assay in the case of control cells and in cells pre-treated with O^6-BG without chloroethylating agent incubation.

Further, we decided to evaluate the contribution of p53 tumor suppressor for the induction of apoptosis by TMZ and BCNU through the nuclear condensation assay.

In HCT116 p53-null cells (Figure 4.7 - A) there was no significant induction of apoptosis in cells with O^6-BG pre-incubation followed TMZ treatment. The same pattern for the induction of apoptosis was obtained for cells incubated with TMZ only. The values obtained for this assay in both conditions were similar to respective controls.

As expected, no variations in percentage of apoptotic cells were detected in control cells and in cells pre-treated only with O^6-BG.

It was verified that, contrarily to TMZ, incubation of HCT116 p53-null cells with BCNU only (Figure 4.7 – B) leads to a significant increase in number of apoptotic cells along the 96h of experiment. However, when cells were subjected to O^6-BG pre-treatment followed by exposure to BCNU agent there was a similar pattern in percentage of apoptotic cells. No significant differences were detectable, at 96h of incubation, by nuclear condensation assay in untreated cells and cells pre-treated with O^6-BG without BCNU agent. Therefore p53 seems to be required for induction of cell death mediated by O^6-chloroethylating agents.



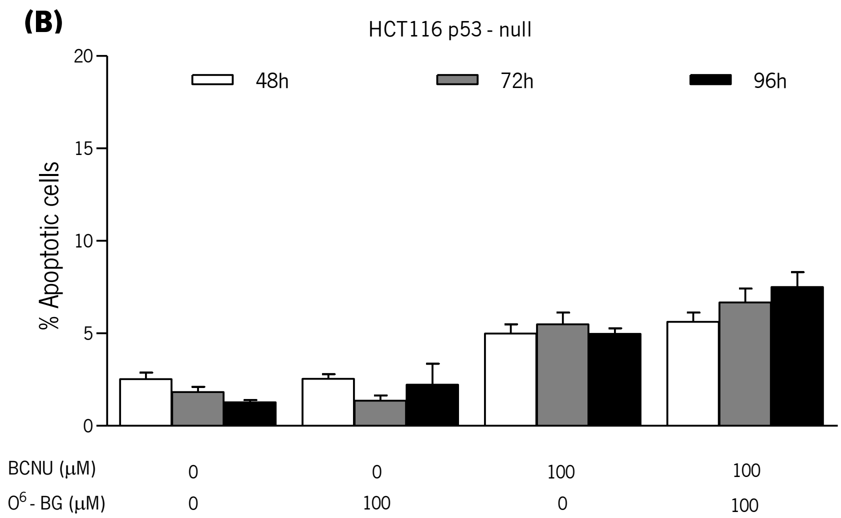


Figure 4.7 – Effect on induction of apoptosis, in HCT116 p53-null cells, by **(A)** TMZ (200μM), O^6-BG (100 μM) or O^6-BG plus TMZ and **(B)** BCNU (100μM), O^6-BG (100 μM) or O^6-BG plus BCNU. Apoptosis was evaluated through nuclear condensation assay at 48, 72 and 96h of treatment. For each condition were performed three independent experiments and data are expressed as mean ± SEM. Significant differences were found, by One-way ANOVA followed by Dunnett's Multiple comparison test, when compared with respective control (untreated cells) (* $P<0.05$; ** $P < 0.01$).

4.6. Effects of some phytochemicals on MGMT protein expression

As already referred, modulation of DNA repair may be assumed to have a relevant role both in the prevention of DNA damage and mutations that may lead to genomic instability and cancer as well as on sensitivity to chemotherapeutic drugs. For instance, an increase of MGMT activity is expected to be a successful chemoprevention strategy. On the other hand, phytochemicals that cause downregulation of this protein can be important to patients undergoing chemotherapy with alkylating agents since this may help improve the effectiveness of the chemotherapeutic agents (Niture et al., 2006a).

We assessed the effects of some natural compounds on MGMT protein expression in Caco-2. For that, after 48h of incubation, the protein levels were monitored by western blot (β-actin antibody was used as loading control). As shown in Figure 4.8, no significant differences on MGMT protein levels are observed in Caco-2 cells between controls and cells incubated with tested phytochemicals (UA, Lut, Q, Curc, Sil, Resv, Rut and AO).

The phenolic acid, rosmarinic acid at 50μM, reduced significantly the expression levels of MGMT protein comparatively with untreated cells. The other phytochemicals had no significant effects on MGMT protein levels.

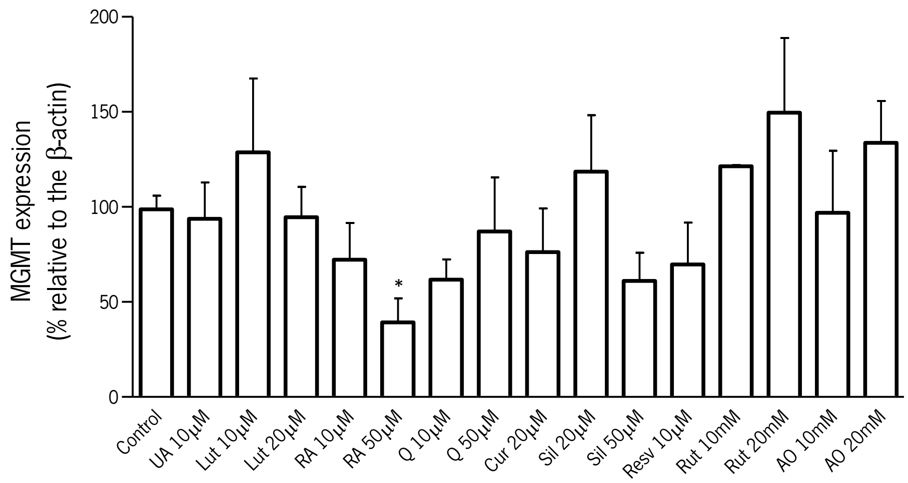


Figure 4.6 – Effect of 48h of incubation with phytochemicals on MGMT protein expression, in Caco-2 cells. The protein levels were evaluated by Western Blot. As loading control, we used β-actin. For each condition three independent experiments were performed and data are expressed as mean ± SEM. Significant differences were found, by Student *t*-test, when compared with control (untreated cells) (* $P<0.05$).

5. DISCUSSION AND CONCLUSIONS

Tumors of the colon and rectum (CRC) comprise a large number of cancers and are responsible for many deaths. The significant resistance to several drugs used in treatment of this type of cancer contributes to the low life expectancy of many CRC patients, a problem that requires new effective drugs and cheap and reliable methods to test them.

Human cells are constantly exposed to DNA damaging agents that are produced endogenously (such as ROS and replication errors) or widely present in the environment which make an important contribution to tumor development. The most important effect of DNA damage is that, without proper repair, they may lead to the accumulation of mutations, genomic instability and cancer.

Due to their cytotoxic effects some alkylating agents are frequently used as chemotherapeutic drugs, with the aim to induce DNA damage and cancer cell death. Based on this, a complete understanding of the cellular elements that determine the biological response to alkylating agents is relevant not only for cancer prevention but also to enhance the efficiency of cancer therapy. The two alkylating agents that were used in the present study, TMZ and BCNU, may cause alkylated base lesions in the DNA template, such as O^6-alkylG lesions (O^6meG and O^6-ClethG, respectively) and *N*-alkylated lesions (N1 and N7 positions of guanine). Consequently, these DNA lesions can be repaired by some DNA repair pathways (mainly by direct repair MGMT, MMR, BER, NER and HR systems) in an attempt protect from genotoxicity and mutagenesis (Fu et al., 2012). Cell death by apoptosis is the desired cellular response after the application of chemotherapeutic alkylating agents to cells. If the DNA lesions are not repaired, they may lead of blockage of DNA replication process causing stalling of replication forks and DSB formation that signal to cell death. Apoptosis following DNA damage have a protective role preventing the mutagenic or carcinogenic events. In addition, deficiencies in the activation of apoptotic response increase cancer incidence and, cause resistance of tumor cells when chemotherapy based on alkylating agents is applied.

As referred before, also the DNA repair pathways modulate the sensitivity/resistance to alkylating agents. The MGMT protein has an important role on repair of initial O^6alkylG lesions induced by both methylating and chloroethylating agents and, therefore, while playing an important role in cancer prevention, MGMT also constitutes the major mechanism of resistance to alkylating chemotherapeutic agents. Therefore, the inhibition of MGMT using pseudosubstrates (namely, O^6-BG) improves the effectiveness to chemotherapy in cancer cells (Ramos et al., 2013; Verbeek et al., 2008). In agreement with this, our results show that, the isolated application of

TMZ or BCNU reduced significantly the cellular viability. However, the combination of O^6-BG with either of the two alkylating agents increased the effectiveness of the therapeutic agent since it reduced drastically the number of viable cells when compared with respective control cells (untreated cells). O^6-BG, therefore, potentiated TMZ and BCNU cytotoxic effects.

Some already available methodologies allow the quantification of MGMT repair activity (Christmann et al., 2011). However, the development of low cost and non-radioactive strategies is still necessary. The comet assay is a simple and low-cost method used for the assessment of DNA damages. Frequently, this method is used for measure the oxidative DNA damages as well as to evaluate the DNA effects of some natural compounds (chemoprevention). However, alkylating damages are more relevant both in terms of carcinogenesis and in the induction of cell death during cancer therapy. In a previous study (Ramos et al., 2013), the comet assay was used to evaluate the O^6meG lesions caused by MNU in MGMT-inhibited and MMR-proficient cells. Here, we tested a new application for the comet assay with regard to estimate of both levels of O^6meG as well as O^6ClethG induced by alkylating agents. TMZ and BCNU were used in the presence or absence of O^6-BG and the conversion of O^6-alkylations into strand breaks by MMR-proficient cells. The concentration of O^6-BG used that inhibited MGMT was previously defined (Ramos et al., 2013). The results obtained in the present study not only confirm that the comet assay can be used to detect O^6meG, as described previously by our group (Ramos et al., 2013), but also show the detection of O^6ClethG lesions induced by chloroethylating agents. As observed in figure 4.2 – A and B, when cells were incubated only with TMZ or BCNU agents respectively, DNA damages were almost completely repaired by the second cycle of replication of Caco-2 cells (at 72h). This is expected because MGMT removes efficiently both initial O^6meG or O^6ClethG lesions introduced in the DNA template. MGMT efficiently transfers the alkyl group introduced in DNA base to the cysteine residue present in its active site (Kaina et al., 2010). However, when MGMT is inhibited by the application of O^6-BG the comet assay detected high levels of DNA damages during the 72h of incubation because unrepaired O^6ClethG or O^6meG remained in the DNA template and are converted to strand breaks (SBs) through to the recognition and action of other proteins that belonging to DNA repair pathways such as MMR and NER during the cell replication (Kaina et al., 2007; Kondo et al., 2010b). Cells treated with O^6-BG plus TMZ treatment, demonstrated an increase in DNA damage at 72h. This effect was not present at 48h because Caco-2 cells have very slow replication cycles and, O^6meG lesions require two cell cycles to be converted into strand breaks. For both alkylating agents with or without the presence of O^6-

BG, at 1h of incubation, significant DNA damages were observed and, correspond to the initial damages (namely AP sites and SSBs) caused by BCNU and TMZ. This fact was in accordance with previous studies that used the methylating agent MNU (Ramos et al., 2013). For instance, BCNU is a chloroethylating agent that besides the O^6ClethG lesions also induces N7-alkylG and N1-alkylG (Cui et al., 2009). These N-alkyl lesions are recognized and efficiently removed from DNA template by the BER system (Stojic et al., 2004). The MPG, a glycosylase that belongs to BER system, promotes the hydrolysis of the *N*-glycosylic bond (after and before of lesion site) which leads to the formation of AP sites (Maynard et al., 2009; Robertson et al., 2009). These AP sites are converted in breaks (Hegde et al., 2008) which allows their detection by the comet assay. However, their complete repair is carried out by other enzymes involved in BER pathway and, their contribution to total damage decreases rapidly due to efficient repair by BER. In agreement with this the methylating agent TMZ, produced similar results after 1h of incubation since this drug leads to the formation of monoadducts such as N^7meG and N^3meG besides the O^6meG (Zhang et al., 2012). Again, these lesions are repaired by recognition and removal through the activation of BER system that leads to AP site formation.

Because DNA strand breaks (detected by the comet assay) are potent inducers of cell death by apoptosis (Quiros et al., 2010), we decided to quantify apoptotic cell death induced by TMZ and BCNU with or without pre-incubation with MGMT inhibitor, O^6-BG. Results showed that after 96h of incubation with TMZ and 72 and 96h of incubation with BCNU (Figure 4.3 – A and B) there was also a significant increase in apoptotic cell death in MGMT inhibited cells. Therefore, our results are in agreement with the findings previously described by others, since, in the case of TMZ, if the MGMT enzyme does not repair all O^6meG lesions (due to, for instance, the inhibition of MGMT through pseudosubstrates), these lesions mispair with thymine, during replication process forming O^6meG-T mismatches. This damage is recognized by MMR proteins that promote the removal of thymine from complementary strand. However, as long as O^6meG remains in one the DNA template, thymine is again inserted and the MMR process will be repeatedly activated, resulting in a futile repair cycle. The recognition by MMR creates single strand breaks in the newly DNA strand that are accumulated and become cytotoxic after some replication cycles (Kaina et al., 2010). After two rounds of replication DSBs may eventually arise (Ochs and Kaina, 2000; Quiros et al., 2010) and they are considered potent activators of the apoptosis (Quiros et al., 2010; Verbeek et al., 2008). After incubation of cells with O^6-BG plus BCNU, the O^6ClethG lesion, an unstable monoadduct also repaired by MGMT remains in the DNA template and is converted into N1-O^6-ethanoguanine (N1-O^6EtG) that is capable to react with cytosine present in complementary strand forming a N1-guanine-N3-cytosine interstrand crosslink. If ICLs are not repaired, through to the repair proteins involved in NER system (Batista et al., 2007), they will lead to the formation of DSBs, upon replication, that induce cell death by apoptosis (Figure 5.1) (Kaina et al., 2010; Roos and Kaina, 2006).

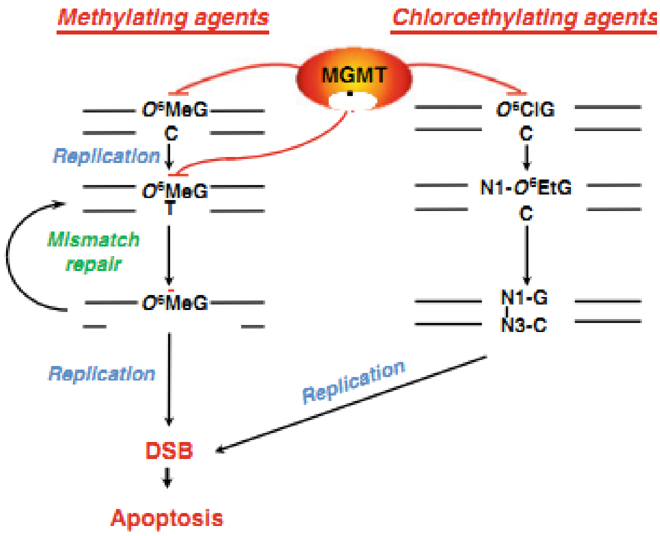


Figure 5.1 – Repair of O^6meG and O^6ClethG lesions is executed by direct action of MGMT. In the absence of MGMT or under MGMT inactivation by O^6-BG, these lesions not are removed from the template and eventually result in DSBs formation that are potent inducers of apoptosis. For apoptosis occurs in TMZ needs that MMR system recognizes DNA lesions and converted them into SBs. On the other hand, chloroethylating agents lead to the formation of ICLs that blocks the replication process causing cell death independently of MMR (Adapted from (Kaina et al., 2010)).

Significant apoptotic cell levels that were detected only after 96h of incubation with O^6-BG plus TMZ treatment are in accordance with other groups' observations. The processing of O^6meG lesions into DSBs requires two replication cycles while the induction of apoptotic process only occurs in subsequent cell replication cycles (Quiros et al., 2010). Another study also demonstrated that, in glioma cells that, O^6meG triggers apoptosis after 120h of incubation requiring extensive cell proliferation (Roos et al., 2007). Non-significant levels of apoptosis were induced by the methylating (TMZ) and chloroethylating (BCNU) agents alone (Figure 4.3 – A and B, respectively) since, as mentioned before, MGMT efficiently repairs O^6meG and O^6-ClethG initial lesions from DNA template and consequently there is no formation of breaks in DNA and hence the apoptotic process was not triggered.

In conclusion, this Cometh methodology allows the assessment of alkylating DNA damages as mentioned before thereby being useful for the screening of new chemotherapeutic drugs that depend on the activity of MGMT protein. In addition it allows the test of other MGMT inhibitors. On the other hand, we verify that the application of O^6-BG improves the effectiveness therapeutic of the O^6-alkylating agents sensitising colorectal cancer cells to cell death. This study also suggested that the methylating and chloroethylating agents induced apoptotic cell death in an O^6-alkylG- dependent manner.

Effect of mismatch repair system on DNA damage and induction of apoptosis by alkylating agents

During the past years, several experiments have evidenced that mismatch repair mechanism are implicated in the cellular response to methylating agents. It has been described that O^6meG does not have the ability to trigger cell death directly. For this, the activation of MMR system is required and MMR-deficient cells have demonstrated to be highly resistant to killing by S_N1-type alkylating agents, such as MNNG and TMZ (Hickman and Samson, 1999; Ochs and Kaina, 2000; Pepponi et al., 2003). However, the contribution of mismatch repair system in chemoresistance to chloroethylating agents (such as BCNU) is still unclear and more studies are required its complete understanding. Therefore, in this part of work we decided to analyze the contribution of MMR system for the recognition of O^6-alkylG lesions and their conversion to SBs. To this end, we measured the DNA damage (Figure 4.4 – A and B) induced by TMZ and BCNU by a CoMeth assay and quantified apoptotic cell death (Figure 4.5 – A and B) using a MMR-deficient cell line (HCT116). We demonstrated that, in HCT116 cells, after 72h of incubation with TMZ or O^6-BG plus TMZ (Figure 4.4 - A) the values obtained for DNA damages were similar to those obtained for control cells (DMSO only). Comparatively with above results, the Caco-2 MMR-proficient cells exhibited significant high levels of DNA damage at 72h after incubation of O^6-BG plus TMZ agent (Figure 4.2 – A). As expected, in HCT116 cells the comet assay did not detect O^6meG lesions because they were not transformed into SBs due to the presence of a defective MMR system. In opposition of Caco-2 cells (Figure 4.3 – A), induction of apoptosis did not occur in HCT116 cells even in the presence of MGMT inhibitor O^6-BG (Figure 4.5 - A). The reason is that O^6meG:T mispairs were not recognized by MMR system and formation of SBs did not occur thereby not triggering cell death by apoptosis. These results corroborate with studies performed previously by our group using MNU methylating agent (Ramos et al., 2013). Taken this into account, it is possible infer that, MMR-deficient cells (HCT116) were resistance to TMZ therapy compared to the MMR-proficient cell line Caco-2. With regard to cell death results, the same observation had already been made by Noonan and collaborators since they demonstrated that TK6 cells (MGMT – and MMR – deficient) did not exhibit significant levels of apoptotic cells following high doses of MNNG treatment when compared with TK6 MGMT- and -MMR proficient cells (Noonan et al., 2012). A possible mechanism is that, in response to DNA damage induced by methylating agents, human MutSα (MSH2/MSH6) and posteriorly MutLα (MLH1/PMS2) are recruited, bind and efficiently recognize O^6meG:T mispairs and, consequently, promote the recruitment of ATR to DNA lesion sites with activation of ATR kinase resulting in phosphorylation of the downstream targets (such as Chk1) (Yoshioka et al., 2006) that trigger several DNA damage responses (DDR) including apoptosis (Jackson and Bartek, 2009). The authors refer that recruitment and activation of several DDR network proteins occurs in a manner that is dependent the presence of a proficient MMR system (Yoshioka et al., 2006). Recently, two models have been proposed that demonstrate the connection between the MMR system and methylating agent sensitivity (Figure 5.2): a direct signaling model and indirect futile repair model. In the direct signaling model the recognition and binding of O^6meG:T mismatches by MMR proteins (MutSα and MutLα) leads to the direct activation of key DNA damage signaling kinases (such as ATR kinase), that in the presence of high levels of these mismatches trigger cell death by apoptosis. The indirect futile repair model proposes that repeated cycles of recognition, excision of the lesion and reinsertion of thymine nucleotide in new DNA strand at O^6meG:T mismatches sites, due to the action of MMR pathway, leads to futile repair cycles and occurrence of SSBs. These SSBs function as an additional substrate for indirect signaling activation. After 2 replication cycles these breaks cause stalled replication forks and consequently lead the formation of DSBs. These events culminate in DDR network activation that promote the phosphorylation of a number of proteins involved, namely ATM, ATR, Chk1 and p53 and induction of apoptotic cell death (Noonan et al., 2012).

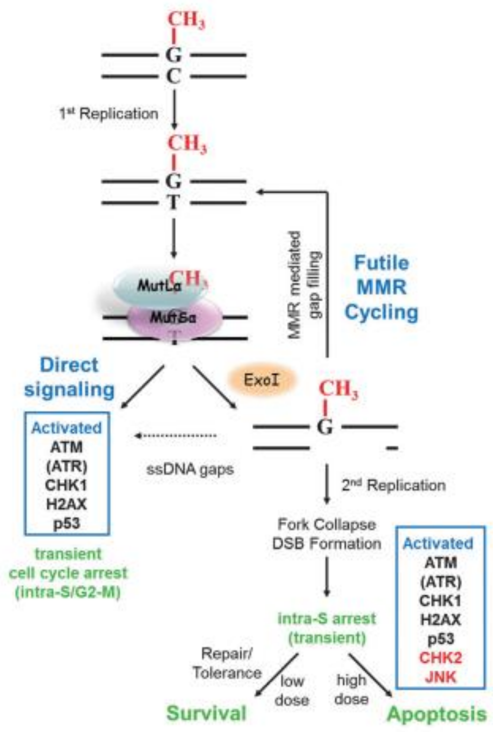


Figure 5.2 – Two models proposed by interaction between MMR system and sensitivity of methylating agents. A direct signaling model (left) and indirect futile repair model (right) may work simultaneously in order to mediate the cellular response to methylating agents (from (Noonan et al., 2012)).

On the other hand, with regard to BCNU treatment there is controversy about the dependence of the MMR system for cytotoxic effects. There are experiments that suggested that MMR system, although weakly, also is involved in the protection of the melanoma cell lines against BCNU treatment (Pepponi et al., 2003). On the contrary, Cui and collaborators demonstrated that DNA damage response caused by BCNU treatment was independent of the functional MMR system status (Cui et al., 2009).

Our results demonstrated that, when BCNU treatment is applied to MMR-deficient cells (Figure 4.4 - B) DNA damages were gradually decreasing during the subsequent 72h of incubation, because, as referred above, MGMT efficiently repaired O^6ClethG lesions from the DNA template. As expected, low levels of apoptotic cells were detected in this condition over the entire time of experiment (Figure 4.5 - B). However, when MMR-deficient cells were pre-incubated with O^6-BG, for MGMT inhibition, there were high significant levels of DNA damages at 72h, as also observed above for the case of MMR-proficient Caco-2 cells (Figure 4.2 - B). The unrepaired O^6-ClethG lesions remain in the DNA strand and rapidly react with cytosine and interstrand crosslinks (ICL) are formed. This leads to the blockage of DNA replication machinery and the

DSBs are created after one cycle of replication (Figure 5.1) (Kaina et al., 2010). As observed in MMR-proficient Caco-2 cells (Figure 4.2 - B), in the presence of O^6-BG plus BCNU the percentage of apoptotic cells was significantly higher compared with control cells (Figure 4.5 - B). By comparing the Caco-2 and HCT116 cells is possible to suggest that the cytotoxic effects induced by BCNU treatment are independent of functional status of MMR system. Recently in a study with medulloblastoma cell lines, it was proposed that DNA interstrand crosslinks are the main responsible for activation of the DDR pathway that culminate in ATR-Chk1 activation (Cui et al., 2009) triggering eventually apoptotic cell death (Kaina et al., 2010). In conclusion, cytotoxicity induced by BCNU may occur in an MMR - independent manner.

In conclusion, the application of BCNU treatment in tumors that have microsattelite instability (MSI) due to the loss of MMR system may be a good alternative to the use of TMZ.

Effect of p53 tumor suppressor on DNA damage and induction of apoptosis by TMZ and BCNU agents

The p53 tumor suppressor has a prominent role in keeping genomic integrity and preventing cancer development. As "guardian of the genome", its major functions are to regulate the apoptosis and cell growth (Menendez et al., 2009). Upon genotoxic stresses, such as cancer treatment based on chemotherapeutic drugs, this protein is activated leading to its translocation to nucleus and transcription of target genes (Chari et al., 2009). However, as mentioned before, there is controversy about the role of p53 in cancer chemotherapy. We intended to clarify the involvement of p53 on DNA damage and induction of apoptosis in colorectal cancer cells in response to alkylating chemotherapeutic agents. For that, we compared two isogenic cell lines – HCT116 p53-wt and HCT116 p53-null. The initial DNA damage detected after TMZ treatment was decreasing over time, in HCT116 p53-null cells, even in the presence of O^6-BG (Figure 4.6 - A), and consequently without induction of cell death by apoptosis (Figure 4.7 - A). As previously demonstrated, similar results were obtained with p53-wt HCT116 cells since also in this cell line no significant increase were found in number of apoptotic cells at 96h of treatment (Figure 4.5 - A). These results allow us to suggest that TMZ cytotoxic effects are independent of p53 status whereas requiring a functional MMR system. In our study, even in the presence of p53-wt, MMR-deficient cells were not capable to activate the apoptotic process because there was no recognition of the O^6meG:T mismatches by MutSα and MutLα proteins. This way, as suggested by Noonan group that used TK6/MGMT proficient and TK6/MMR deficient cells (Noonan et al., 2012), ATR-Chk1 signaling pathway is not activated and low levels of phosphorylation of Chk1 and p53 were detected. In general, the treatment with methylating agents results in the phosphorylation of p53, however, these phosphorylation depends on the presence of functional MutSα and MutLα proteins in the DNA lesion site (Duckett et al., 1999).

In the case of BCNU treatment, in both cell lines studied (HCT116 p53-wt Figure 4.4 – B and HCT116 p53-null Figure 4.5 - B) it has verified that in presence of O^6-BG, BCNU incubation results in a high levels of DNA damages over time independently of the presence of functional p53 protein. Therefore, these results suggest that the O^6ClethG lesions remain in DNA template forming interstrand crosslink. These ICLs, during the G1 phase of cell cycle are recognized and removed through to nucleotide excision repair. However, if this O^6ClethG lesion cannot be repaired its conversion in DSBs occurs (Deans and West, 2011) allowing the detection by comet assay.

The observations based on nuclear condensation assay showed that BCNU induced apoptosis both in HCT116 p53-wt (Figure 4.5 - B) and HCT116 p53-null cells (Figure 4.7 – B). These results are in agreement with the DSB detected by comet assay. However, when both cell lines were subjected to O^6-BG pre-treatment followed BCNU were found some differences between the two cell lines. It is observed that p53-wt cells were more sensitive than p53-null cells to apoptosis since a higher percentage of apoptotic cells were observed when p53 was functional. Our results are in accordance with other in glioma cells (Hermisson et al., 2006). The induction of apoptosis in both cases is presumably due to the fact that p53-null cells trigger cell death independently of p53 transcriptional activity (Goldstein et al., 2008). Therefore, our results may suggest that O^6ClethG lesion, just like O^6meG (Kaina et al., 2007), may evoke the two apoptotic pathways – mitochondrial- or -extrinsic apoptotic pathway (Fas/CD95/Apo-1) depending on p53 functional status. However, this hypothesis needs to be clarified in subsequent studies in colorectal cancer cells.

For instance, after O^6-ClethG lesion formation it was verified in previous studies that glioma cells with mutated *p53* gene trigger the activation of mitochondrial apoptotic pathway (Batista et al., 2007) with cells exhibiting a decline in anti-apoptotic Bcl-2 protein levels leading to the release of cytochrome c from mitochondria. This release activates Bax and Bak pro-apoptotic proteins and pro-caspase 9. Meanwhile, this pro-caspase promotes the activation of the effector caspases, such as caspase 3 and 7 (Hector and Prehn, 2009). On the other hand, the same study also revealed that in cells with wt-p53, O^6-ClethG lesions lead to the activation of both extrinsic apoptotic pathway (by stimulation of death receptor on the cell membrane, FasR) and the intrinsic apoptotic pathway (Batista et al., 2007). In this pathway, the FasR and its respective ligand (FasL) promote the induction of caspase-8 which in turn activates their effector caspases (such as caspase 3) (Hector and Prehn, 2009). This is also in line with some other studies with methylating treatment, where wt-p53 cells underwent apoptosis through the extrinsic apoptotic pathway (Dunkern et al., 2003; Roos et al., 2004; Roos et al., 2007) and in p53 mutated cells where there was the activation the mitochondrial apoptotic pathway (Figure 5.3) (Ochs and Kaina, 2000; Roos et al., 2007).

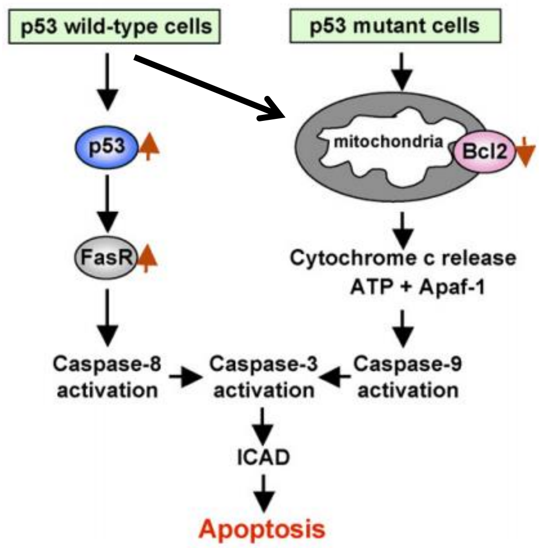


Figure 5.3 – Apoptotic pathways triggered by O^6-alkylG lesions in cancer cells with wildtype p53 (left panel) or with mutated p53 tumor suppressor (right panel) (Adapted from (Kaina et al., 2007)).

Due to the role of p53 in chemosensitivity will be fundamental to explore if the same apoptotic behavior dependent on p53 status, demonstrated in glioma cells, also applies to colorectal cancer cells.

Therefore, the determination of p53 status in tumors will be useful to predict the response to alkylating agents in colorectal cancer patients.

Impact of natural compounds on MGMT protein expression

MGMT is very relevant since it performs not only functions in cancer response to chemotherapy based on alkylating agents but also function in cancer prevention. Dietary natural compounds that promote an increase of MGMT activity levels may be useful in terms of chemoprevention. In turn, downregulation of MGMT activity through the use of natural compounds may be beneficial for cancer therapy based on alkylating agents. Our results of natural compounds on expression of MGMT by western blot showed that rosmarinic acid (RA) may contribute to increase the cytotoxicity induced by alkylating agents since it induced a decline in MGMT expression levels (48h of incubation with RA (50µM)) (Figure 4.8).

Although this still needs to be verified, daily consumption of RA may be beneficial when patients are subjected to cancer therapy based on alkylating agents, since it might raise treatment efficacy and may be reduce the side effects associated with therapy.

Concluding remarks

With this project it was possible demonstrate that the comet assay (CoMeth) also can be efficiently used to assess alkylating DNA damages, O^6ClethG, induced by chloroethylating agents, BCNU in addition to O^6meG induced by TMZ. It was possible to observe that the pre-incubation of cells with MGMT inhibitor, O^6-BG, potentiates the cytotoxic effects caused by these two alkylating agents. This method will allow test new MGMT inhibitors, evaluate the potential effects of other chemotherapeutic agents that depend by the action of MGMT activity. Furthermore, it will may also allow testing compounds (for instance, demethylating agents) that enhance the expression of MGMT and MMR genes (that are often epigenetically silenced in several tumor types).

We found that for methylating agents exert their cytotoxic effects in cancer cells they require the presence of a functional MMR system for conversion of O^6meG lesions into strand breaks (detected by the comet assay when MGMT is inhibited). When this DNA repair system not is functional, cancer cells showed a higher resistance to TMZ treatment since apoptosis did not occur. Therefore, CRC MSI patients need alternative treatment that based on drugs that do not require the functional status of MMR system. Contrary, the obtained results suggest that DNA damages and cell death by apoptosis induced by BCNU occurs in an MMR-independent manner. We can suggest that the application of BCNU to patients with microsatellite instability (MSI) due to loss of MMR system may be a good alternative for therapeutic strategy.

We then investigated the influence of p53 status in sensitivity to alkylating drugs by comparing effects in two isogenic cell lines, HCT116 p53-wt and the same line knockdown for p53 (HCT116 p53-null). The results suggest that the absence of p53 tumor suppressor gene attenuates BCNU cytotoxicity (lower percentage of apoptotic cells), when O^6-BG was applied on colorectal cancer cells and did not improve the response to TMZ in MMR deficient cells. Collectively, the determination of p53 status as well as MMR will help to identify CRC patients which will be more susceptible to particular alkylating anticancer agents.

In terms of the diet, a phenolic compound present in plants, rosmarinic acid (RA), decreased the MGMT protein expression levels and may be important to cancer chemotherapy based on drugs that depend on the action of direct repair by MGMT.

Although more studies are needed, this result may indicate that the consumption of this compound during the chemotherapeutic process can enhance the efficiency of alkylating compounds.

CoMeth is a useful tool in the test of new chemotherapeutic compounds such as alkylating agents, MGMT inhibitors, p53 regulators, MMR inducers (epigenetic drugs). This cheap and versatile method may also use in nutraceutic research allowing to test chemopreventive natural compounds or other that improve chemotherapeutic sensitivity.

Future perspectives

With this study, we found that methylating (TMZ) and chloroethylating (BCNU) agents induced DNA damages that are detected by the new methodology previously developed in our group. These lesions after the intervention of some DNA repair systems are converted in DSBs that may culminate in cell death by apoptosis. For comprove the results, in future work, we would compared the effects 5-fluoropyrimidine 5-fluorouracil (5-FU) and Oxaliplatin, that are the standard therapies applied in cases of CRC patients, and their dependence of MMR system for caused DNA damages, using comet assay, and apoptotic cell death.

Another topic that would be interesting to investigate is concomitant application of natural compounds and alkylating agents in colorectal cancer cells. Considering the potential effects of dietary compounds on cancer prevention, studies that evaluate the role of diet during chemotherapy process are needed. Therefore, will be interesting try to understand if the consumption of dietary compounds influences the response of colorectal cancer cells when is being treated with alkylating agents. For that, parameters such as DNA damage, by comet assay, and cell death by apoptosis, using nuclear condensation assay, would be evaluated.

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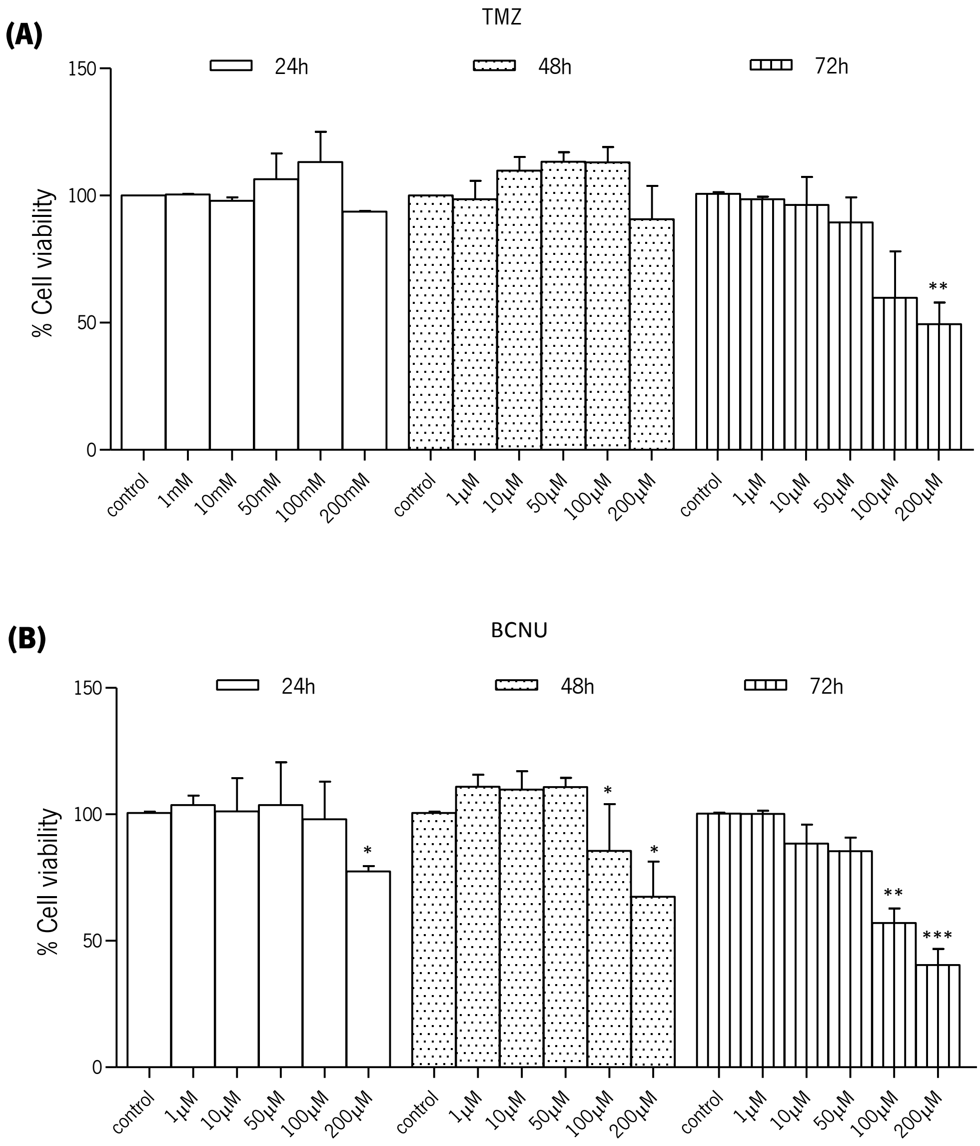
7. ANNEXES

In order to define the adequate concentration that are able to reduce the cell viability to about 50% was performed a MTT assay. Cells were treated with TMZ (Figure 1- A) or BCNU during 24, 48 and 72h with defined concentrations (range between 1μM - 200μM).



Supplementary Figure 4.3 – Dose dependent influence on cellular viability in Caco-2 cells of **(A)** TMZ and **(B)** BCNU, by MTT assay. Different concentration of TMZ and BCNU were incubated in cells after 24, 48 and 72h. Each condition represents mean ± SEM for three independent experiments. Significant differences, determined through Student's *t*-test, were found compared with control cells (*P < 0.05; *** P < 0.001).

The results demonstrated that cell viability decreased with increasing concentrations of TMZ (supplementary Figure 3.1 – A) and BCNU (supplementary Figure 3.1 – B) in Caco-2 cells. The Caco-2 cells seem to be more susceptible to methylating agent only after 72h where there is a reduction of 50% in cell viability. The same conclusion can be applied for case of BCNU agent.

Lysis buffer for comet assay

To prepare one liter of Lysis buffer, 1.211g of Tris Base (10mM), 146.1g of NaCl and 37.22g of Na_2EDTA were dissolved in 850ml of water. Posteriorly, the pH is adjusted to 10 using NaOH. To complete the buffer solution, it is also necessary to add 1% (v/v) of Triton X-100.

Electrophoresis buffer for comet assay

To prepare electrophoresis buffer is necessary dissolved, in 800ml of distilled water, 100ml of NaOH (300mM) and 5mL of EDTA (200mM). After dissolution the volume was adjusted to 1L.